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INTRACELLULAR ENZYMES

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A COURSE OF LECTURES GIVEN IN THE PHYSIOLOGICAL LABORATORY UNIVERSITY OF LONDON

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PREFACE

THE subject of these lectures might at first sight be regarded as too small and unimportant to warrant their reproduction in book form, but I hope that such an opinion may be dispelled by a study of the lectures themselves. The progress of research renders it more and more evident that the cellular protoplasm of all living organisms is made up very largely of ferments or enzymes, and that many or most of its properties are dependent upon their activities. The literature dealing with these intracellular enzymes is scattered and somewhat fragmentary, and comparatively little of it has as yet found its way into text-books. This is partly because of its recent origin, for reference to the authorities cited at the foot of these pages will show that almost the whole of the research work described has been carried out during the course of the last decade. If such rapid rate of progress be continued in the future, the subject of intracellular enzymes bids fair to become, if it has not already become, one of the most important branches of biochemistry, for it alone seems to offer a clue to the solution of the most fundamental of all biological problems, the nature and constitution of protoplasm.

The matter in this book closely follows that of the spoken lectures, with some amplification of detail. I take this opportunity of thanking Dr A. D. Waller for his kindness in inviting me to give the course of lectures in the Physiological Laboratory of the University of London, for I should scarcely have had the energy to collect and publish the material without the stimulus of such an invitation. Also, I am indebted to Dr W. M. Bayliss for his kindness in looking through the MS., and offering valuable criticism. H. M. V.

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INTRACELLULAR ENZYMES

LECTURE I

PROTEOLYTIC ENDOENZYMES

Dependence of chemical activities of living tissues on endoenzymes. Liberation of endoenzymes on death of cells: gradually, if tissues be kept intact; immediately, if minced up. Methods of extracting endoenzymes. Classification of proteolytic endoenzymes. Proteases, endoerepsins, arginase, urease. Action of enzymes on polypeptides. Amide nitrogen in relation to enzymes and acids.

OF the numerous subjects included under the head of Physiological Chemistry, or Bio-Chemistry, few have attracted so much attention within recent years as that of Enzymes. And great as has been the increase in our knowledge of the nature and mode of action of these substances, the further we advance the wider becomes the field of research opening out before us. This is especially true in respect of the group of enzymes known as Intracellular or Endo-enzymes. These enzymes differ from the exo-enzymes, such as are found in many of the secretions of living organisms, by reason of the fact that they are bound up in the protoplasm of the cells, and, so long as these cells retain their vitality, can only exert their activity intracellularly. On death of the cells, the protoplasm disintegrates, and many of the constituent enzyme groupings gradually split off and pass into solution. It is inferred, though strict proof of the inference is wanting, that any zymolysing powers possessed by such solutions were, in all probability, possessed by the protoplasm before disintegration. And as a living tissue would scarcely elaborate and store up within itself enzymes which were useless to it, it is supposed that any enzyme which can be extracted from a tissue after death—apart from

such enzymes as may be secreted externally during life—was of functional importance during the life of the tissue. A thorough study of all the zymolysing powers possessed by the disintegration products of various typical tissues, vegetable as well as animal, is therefore of paramount importance, for the knowledge so attained may lead us far towards the explanation of the properties of living matter. It is possible that it may show us that many or most of the katabolic processes of living tissues, and perhaps the anabolic processes as well, are due to nothing more than the ceaseless activity of a vast variety of endoenzymes, bound up together in the biogens, and exerting their powers as they are needed. Thus Duclaux suggested that the life of bacteria is nothing more than the sum total of the activities of the enzymes contained within them, and Hofmeister[1] supposed that the multifarious activities of the liver cell, such as the synthesis and hydrolysis of glycogen, the formation of bilirubin and bile acids, of ethereal sulphates and urea, merely represent the action of the different enzymes contained within it. This hypothesis of cellular metabolism is not at present by any means completely established on a sound experimental basis, but it is at least a working hypothesis, and one which can only stimulate research, not retard it. Hence, even if it ultimately prove erroneous, it needs no further justification. It is from the point of view of the probable validity of this hypothesis that the experimental data collected together in these lectures are described.

To pass from theory to fact, it is first necessary to mention briefly the methods used for extracting endoenzymes from the tissues. These enzymes are fixed by some definite chemical bond in the cellular protoplasm, and even after death they are, as a rule, only set free very gradually, provided the tissue be left intact. This is well shown by perfusing an excised organ (*e.g.*, a mammalian kidney) for some days with an antiseptic medium, such as 2 per cent. NaF solution. Even after six days' continuous perfusion, the amount of endoenzymes washed out is so small that it can scarcely be estimated; but a sudden change in the conditions of perfusion, such as the substitution of

[1] Hofmeister, *Die chemische Organisation der Zelle*, Brunswick, 1901.

chloroform saline for the sodium fluoride, causes an immediate and very rapid setting free of the endoenzymes.[1]

Another and simpler method of measuring the autolysis of intact tissues is to keep the organ under investigation in a moist chamber for the required time, and then wash out the autolytic products with saline solution. The results obtained in three experiments with rabbits' kidneys are given in the table. A kidney kept for three days at 14° C., yielded 12 units of erepsin during the first four hours' perfusion, and 82 more during the next six days, whilst at the end of this time it still retained 18 units bound up in the tissues. Two other kidneys, kept for seven and eight days respectively before perfusion, yielded three to five times as much erepsin during the first

Condition of Kidney.	Number of Days Perfused.	Units of Erepsin washed out during			Erepsin remaining in Kidney at end of Perfusion.
		0 to 1 hour.	1 to 4 hours.	4 hours onwards.	
Kept 3 days at 14°	6	6	6	82	18
„ 7 „ 12°	7	43	22	61	15
„ 8 „ 15°	4	28	10	85	24
Fresh . . .	7	·5	·7	87	1
„ . . .	6	·8	·5	62	1
„ . . .	5	·5	...	188	6

four hours' perfusion, but in spite of this, and of a subsequent four to seven days' perfusion, they still retained a fair amount of the enzyme bound up in their tissues. The amount so bound up after a long-continued perfusion is very variable, and it will be seen that the three kidneys which were perfused immediately after excision, and which yielded very little enzyme during the first four hours' perfusion, retained very little after five to seven days' perfusion under various abnormal conditions as of putrefaction and of chloroform treatment.

When organs and tissues are disintegrated by mechanical means, the endoenzymes break free from their anchorage and pass into solution very much more rapidly. If a tissue be finely minced or chopped with a knife, and an extracting medium be added, a large portion of the endoenzymes pass into solution at once, but it is a matter of days or weeks before filtered samples

[1] Vernon, *Zeit. f. allgem. physiol.*, 6, p. 399, 1907 ; see also, Lect. viii., p. 199.

of the extract show their maximum zymolysing power. For instance, I found[1] that extracts of most animal tissues, made by adding two parts of glycerin to one part of chopped tissue, attained their maximum ereptic activity in about three weeks. If, on the other hand, the extracting medium consisted of a mixture of three parts of glycerin and two parts of water, the maximum activity was attained in four or five days.[2]

If the state of division of the tissue particles be fine enough, it is probable that most of the endoenzymes are set free immediately, and pass into solution. Hence, if sufficient material is available, the method first adopted by Buchner[3] for obtaining endoenzymes should be used. This method consists in mixing the tissue—or micro-organisms—with an equal weight of quartz-sand, and grinding up the mixture till the cells are thoroughly broken up. If the mass becomes too liquid, some of the moisture-absorbing siliceous earth, *kieselguhr*, should be added in addition. The mass of disintegrated tissue and sand is wrapped up in hydraulic chain-cloth, and by means of a hydraulic press subjected to considerable pressure. The juice of the broken-up tissue cells is thereby squeezed out, and this juice probably contains the major part of the soluble endoenzymes. But it has been shown by Dauwe[4] that kieselguhr is capable of taking up enzymes, whilst Hedin[5] finds that it has a specific absorption power. For instance, if mixed with a solution of spleen enzymes, it may absorb and remove from solution more than half of the α-protease (an enzyme digesting in alkaline solution), but leave the β-protease (which digests in acid solution) almost untouched. In order to obtain the maximum yield of endoenzymes, it would probably be best, therefore, to avoid using kieselguhr altogether, and keep to sand alone. Also, in the case of soft tissues, such as most animal tissues, the preliminary grinding with sand is unnecessary, as the cells are sufficiently broken up by the impact of the sharp sand grains upon them in the hydraulic press. In the case of micro-organisms, the mechanical disintegration is best effected

[1] Vernon, *Journ. Physiol.*, 32, p. 34, 1904. [2] *Ibid.*, 33, p. 92, 1905.
[3] E. Buchner, *Ber.*, 30, p. 117, 1897.
[4] Dauwe, *Hofmeister's Beitr.*, 6, p. 427, 1906.
[5] Hedin, *Biochem. Journ.*, 2, p. 112, 1907.

by placing the mixture of organisms and sand in a metal cylinder surrounded by a jacket through which cold brine is circulated. A steel axis provided with a series of horizontal vanes is rotated rapidly (up to 5000 revolutions per minute) in the mixture, and the violent intercollision of the sand particles and micro-organisms results in a very complete disintegration.[1] The alternative method adopted by Macfadyen and Rowland,[2] and one suitable for animal tissues as well as micro-organisms, is probably the best of all if expense be no object. It consists in triturating the organisms at the temperature of liquid air (−180° to −190° C.). At this low temperature, the cells become so brittle that no sand need be added to assist disintegration. Also, all chemical decomposition processes are for the time being completely prevented.

It is to be borne in mind that the methods just described can only afford information concerning the *soluble* and fairly stable endoenzymes of the tissues. As will be pointed out in subsequent lectures, there is very little doubt that insoluble endoenzymes exist in addition, and probably other endoenzymes which are so unstable as to lose their activity directly they break free from the tissues.

Another convenient method for obtaining intracellular enzyme preparations is that employed by Wiechowski.[3] The organ is chopped up very finely, and the tissue pulp, mixed with toluol, dried in thin layers on glass plates in a warm room. The dried tissue is then ground up thoroughly, washed with toluol, and is ultimately obtained in such a fine state of division that an aqueous suspension of it is completely filterable through filter paper, and on standing only very slowly forms a deposit. Wiechowski claims that this organ powder contains the tissue proteins and ferments in an uninjured condition.

Proteolytic Endoenzymes.—We now pass on to a description of the more important endoenzymes hitherto identified. First and foremost come the proteolytic endoenzymes. All living matter is continually breaking down its protein constituents and excreting protein disintegration products. Hence, if any or all

[1] *Cf.* Macfadyen, Rowland, and Morris, *Proc. Roy. Soc.*, 67, p. 250, 1900.
[2] Macfadyen and Rowland, *Centralb. f. Bakt.*, 30, p. 753, 1901.
[3] Wiechowski, *Hofmeister's Beitr.*, 9, p. 232, 1907.

of the processes of degradation occurring during life are the work of endoenzymes, we should expect to be able to demonstrate the existence of such enzymes in the tissues after death. In the study of endoenzymes, we should most appropriately examine those of the lowest and simplest of living organisms first, and pass thence to those of the higher animals; but in the present state of our knowledge this is not advisable. Almost all the work hitherto done upon proteolytic endoenzymes concerns mammalian tissues, and so this will be described first.

The existence of proteolytic endoenzymes was first established in 1890, when Salkowski[1] showed that liver and muscle, if minced and kept in chloroform water, underwent what he termed "autodigestion." The data given in the table show the result of the autodigestion of dog's liver. The gland was chopped up, and half of it was placed in ten times its volume of chloroform water, and kept for sixty-eight hours at blood heat. The digest was then filtered, boiled to remove coagulable protein, again filtered, and analysed. By comparison with the control

Soluble Constituents from 1000 gms. of Liver.	Control Experiment.	Chief Experiment.
	Gms.	Gms.
Organic Substances	33·7	46·0
Nitrogen (calculated as Protein) . . .	19·7	39·0
Phosphoric Acid	1·36	1·96
Purin Bases	1·10	1·22

experiment, in which the other half of the chopped-up liver was first sterilised by a current of steam for an hour and a half, and was then incubated in chloroform water, we see that the autodigestion caused a large amount of organic substances to pass into solution. Probably these consisted mostly of protein decomposition products, for the digestion liquid of the chief experiment gave no distinct biuret test, but yielded a considerable quantity of leucin and some tyrosin. The liquid of the control experiment, on the other hand, gave a biuret test, and yielded no leucin. Judged by the increase of phosphoric acid and purin bases, there must have been some autodigestion of the

[1] Salkowski, *Zeit. f. klin. Med.*, 17, p. 77 (suppl.), 1890.

nucleoprotein constituents of the tissues as well as of the proteins. The autodigestion, or, to adopt Jacoby's term, the autolysis, was undoubtedly the work of endoenzymes and not of bacteria, as complete sterility was maintained throughout the experiment.

The increase of purin bases found by Salkowski had been noticed by Salomon[1] nine years previously. He found that the amount of purins in muscle and liver kept at room temperature was doubled during the first twenty-four hours after death. This purin formation he attributed to the action of a ferment set free at the time of death of the tissue cells. He thus foreshadowed the more complete and extensive researches of Salkowski. However, these researches were lacking in one particular, and this was supplied by Schwiening[2] two years later. Schwiening showed that filtration of the liver extracts did not prevent autolysis: in other words, that the autolysis was the work of soluble endoenzymes, and was not determined by insoluble cell constituents. Schwiening also showed that alkalis paralysed the autolysis, whilst acids did not.

In all probability, the autodigestion occurring in tissue extracts and juices is the work not of one or two, but of many endoenzymes. But little attempt has been made to isolate these several ferments, but their existence is clearly indicated by the differential actions of extracts of various organs upon different proteins and protein decomposition products. They are best classified according as they have power to hydrolyse:

(*a*) Native proteins (as fibrin, albumins, globulins).

(*b*) Proteoses, peptones, and polypeptides.

(*c*) Individual amino acids (as glycocoll, hippuric acid, arginin).

(*d*) Urea.

Endoenzymes of the first class are somewhat poorly developed, and the most active press juice obtainable from mammalian tissues is weak in comparison with gastric juice or activated pancreatic juice, or with extracts of gastric mucous membrane or pancreas. At least this is the case if the action

[1] Salomon, *Arch. f.* (*Anat. u.*) *Physiol.*, 1881, p. 361.

[2] Schwiening, *Virchow's Arch.*, 136, p. 444, 1894.

of the press juice upon protein from external sources be tested. Boiled fibrin and coagulated egg white are very slowly attacked, whilst unboiled fibrin passes into solution only after some hours. As a rule, therefore, the action of the endoenzymes upon the native proteins already present in the press juice is investigated, and the rate of conversion of these proteins into non-coagulable proteoses, peptones, and amino acids determined. Any or all of the stages of digestion can be investigated by making Kjeldahl determinations of the total nitrogen in the filtrate from samples of the press juice which have been precipitated by suitable reagents, and comparing them with the nitrogen in the unprecipitated juice. Hot trichloracetic acid precipitates native proteins, but not proteoses, peptones, or amino acids. Saturated zinc sulphate, and 7 per cent. tannic acid precipitate proteoses, but not peptones or amino acids, whilst a mixture of 40 per cent. phosphotungstic acid with 10 per cent. sulphuric acid precipitates proteoses, peptones, and di-amino acids such as hexone bases and cystin, but not mono-amino acids.

Working with such precipitants, Hedin and Rowland[1] demonstrated the existence of a fairly active β-protease enzyme in the expressed juice of a number of organs. They kept the

	Nitrogen at or beyond the Peptone Stage.			
	Before Digestion.	After 16 hours.	After 22 hours.	After 40 hours.
Spleen Juice alone	7·2	17·4	17·6	19·8
„ +·25% Acetic Acid .	...	26·7	27·8	30·0
„ +·1% HCl . . .	...	25·0	27·8	30·2
„ +·37% Na_2CO_3 . .	...	9·6	9·4	10·8
Boiled Juice + ·25% Acetic Acid . .	7·4	7·6	...	...

juice at body temperature in the presence of toluol, and at fixed times precipitated samples of it with 7 per cent. tannic acid, and estimated the nitrogen in the filtrate. Spleen juice and kidney juice proved to be the most active of all, and some of the data obtained with ox spleen juice are given in the table. The total nitrogen in 5 c.c. of the juice corresponded to 35·1 c.c. of $N/10$

[1] Hedin and Rowland, *Zeit. f. physiol. Chem.*, 32, pp. 341 and 531, 1901.

acid, and we see that 7·2 parts of this nitrogen was at or beyond the peptone stage before digestion began. After forty hours, 19·8 parts of it had got to this stage, but when acetic acid or hydrochloric acid was added to the juice, the hydrolysis was so much accelerated that 30·0 parts (or 85 per cent. of the whole) reached this stage. The β-protease is, in fact, an acid-acting ferment, and the moderate digestion occurring in the unacidified juice is dependent on this juice having a considerable natural acidity (corresponding in the present instance to $N/40$ NaOH). Slight over-neutralisation of the juice with sodium carbonate almost stopped the autolysis. Such as did occur was probably due to another endoenzyme, called by Hedin α-protease, which digests in alkaline solution.

Juice of Skeletal Muscle of Horse. (Total N = 56·3.)	Nitrogen at or beyond Peptone Stage.			
	Before Digestion.	After 2 days.	After 5 days.	After 1 month.
Juice alone	17·8	33·4	36·5	51·4
„ +·25% Acetic Acid . .	...	30·6	33·8	45·0
„ + $CaCO_3$ . . .	...	31·2	37·1	53·4

The press juice of lymphatic glands was found to be almost as active as that of the spleen and kidneys, whilst that of the liver was distinctly less active. That of heart muscle was less active still, and weakest of all was the juice of skeletal muscle. The best results obtained with this juice are given in the table,

Juice of Heart of Ox. (Total N = 35·9.)	Nitrogen at or beyond Peptone Stage.			
	Before Digestion.	After 16 hours.	After 3 days.	After 15 days.
Juice alone	9·6	10·5	13·2	17·4
„ +·25% Acetic Acid . .	...	13·0	17·9	22·6
„ + $CaCO_3$ . . .	...	10·3	11·8	13·9

and they show that the protease acted better in neutral solution (neutrality being effected by addition of excess of calcium carbonate) than in acid solution. That of heart muscle acted

best in acid solution, but neutralisation with $CaCO_3$ or MgO did not diminish the relative rate of autolysis to so great an extent as in the case of spleen, kidney, and liver juice. We must therefore conclude, either that the proteases of one organ differ in kind from those of another, or that the relative amounts of acid-acting and alkali-acting proteases vary considerably.

The influence of acids and alkalis upon tissue autolysis has been studied by Wiener,[1] Baer and Loeb,[2] Preti,[3] and Arinkin,[4] and their observations confirm the observations of Hedin and Rowland. Arinkin investigated the influence of various acids upon liver autolysis, and he found the optimum acidity to be ·056 per cent. of HCl, ·075 per cent. of H_2SO_4, ·10 per cent. of H_3PO_4, and ·277 per cent. of lactic acid. This optimum acidity concerns only the hydrolysis of the protein constituents of the tissues, as the nuclein autolysis of the tissues is diminished by the acidification.

A feeble alkali-acting protease, or α-protease, has been isolated by Hedin[5] from the residue left by digesting minced spleen with ·1 per cent. acetic acid. This residue is extracted with 5 per cent. NaCl, and the saline solution dialysed for one or two days. The very scanty precipitate thrown down contains an enzyme which is fairly active when dissolved in ·25 per cent. Na_2CO_3. The slight action which it showed when in acid solution was probably due to the presence of some β-protease as impurity The feeble action of the α-protease is perhaps dependent in part on the presence of an anti-body, for Hedin[6] made the curious discovery that if fresh spleen pulp were kept for twenty-four hours in presence of ·2 per cent. acetic acid, and were then made alkaline and allowed to act upon a suitable protein such as 2·5 per cent. casein solution, it digested it more rapidly than if it had not previously been treated with acid. For this and other reasons he concluded that the acid treat-

[1] Wiener, *Centralb. f. Physiol.*, 19, p. 349, 1905.
[2] Baer and Loeb, *Arch. f. exp. Path.*, 53, p. 1, 1905 ; Baer, *ibid.*, 56, p. 68.
[3] Preti, *Zeit. f. physiol. Chem.*, 52, p. 485, 1907.
[4] Arinkin, *ibid.*, 53, p. 192, 1907.
[5] Hedin, *Journ. Physiol.*, 30, p. 155, 1904.
[6] Hedin, *Hammarsten's Festschrift*, Upsala, 1906.

ment had destroyed an anti-ferment present in the spleen pulp.

Of the two endoenzymes, the α-protease shows considerable resemblance to a weak tryptic ferment, but the β-protease is evidently quite different from both trypsin and pepsin. Like pepsin, it acts in an acid medium: like trypsin, it hydrolyses native proteins to proteoses, peptones, and amino acids. The formation of proteoses in the autolysis of tissues was first demonstrated by Biondi[1] for calf's liver. However, Biondi failed to find either peptones or tryptophan among the autolytic products, and so concluded that the course of hydrolysis is different from that effected by trypsin. Jacoby[2] failed to find either proteoses or peptones in liver autolyses, but in addition to leucin and tyrosin he proved the presence of tryptophan, and likewise of glycocoll, hippuric acid, and urea. In ox spleen juice which had been incubated for two to four months in presence of toluol, Leathes[3] found tryptophan and traces of proteoses, and he isolated leucin, tyrosin, amino-valerianic and aspartic acids, arginin, histidin, and lysin. From a filtered aqueous extract of minced kidney, which had been allowed to digest itself at 36° in presence of ·2 per cent. acetic acid, Dakin[4] isolated all of the products obtained by Leathes except arginin and aspartic acid, and in addition found alanin, α-pyrrolidin carboxylic acid, phenyl-alanin, cystin, and hypoxanthin. Both Leathes and he conclude that the products of action are the same as those formed by trypsin in alkaline media, or those formed by the hydrolytic action of mineral acids, but their results agree with Biondi's conclusion that the course of hydrolysis is different from that effected by trypsin, for neither of them found any peptone. Umber[5] likewise failed to find peptone in the ascitic fluid of two patients examined by him, though he found proteoses, leucin, tyrosin, and traces of hexone bases. We may assume, therefore, not that no peptone is formed at all by the hydrolysis of the

[1] Biondi, *Virchow's Arch.*, 144, p. 373, 1896.
[2] Jacoby, *Zeit. f. physiol. Chem.*, 30, p. 149.
[3] Leathes, *Journ. Physiol.*, 28, p. 360, 1902.
[4] Dakin, *ibid.*, 30, p. 84, 1904.
[5] Umber, *Münch, Med. Woch.*, 1902, No. 28.

proteoses, but that when formed it is immediately split up further into amino acids. Endoenzymes in this respect differ considerably from trypsin, which, though it quickly splits up some of the peptones formed by the hydrolysis of proteoses, has little if any action on others of them (*e.g.*, the so-called anti-peptone of Kühne). The powerful peptone-splitting action of endoenzymes is probably to be attributed, not to the α- and β-proteases mentioned above, but to the erepsin-like enzymes described in the next section.

The action of Hedin's α-protease was examined more in detail by Cathcart,[1] who digested coagulated blood serum with it for seven and a half months at 37° in presence of ·25 per cent. Na_2CO_3 and toluol. The digest showed a faint biuret reaction, and a well-marked tryptophan reaction, and was found to contain leucin, amino-valerianic acid, alanin, phenyl-alanin, tyrosin, α-pyrrolidin-carboxylic acid, lysin, histidin, and arginin. It differed from the acid digest examined by Leathes in containing little or no aspartic acid, but a large amount of glutamic acid, and in the fact that the arginin was optically inactive, instead of being of the usual dextro-rotatory form.

Erepsin.—In 1901 Cohnheim[2] found that if the press juice of intestinal mucous membrane were allowed to act upon proteoses and peptones, these bodies disappeared, just as Hofmeister[3] found that they disappeared from the intestinal mucous membrane of an animal after death. This disappearance was attributed by Hofmeister to the synthetic action of the still living intestinal cells, but Cohnheim found that in his mixtures of press juice and peptones there was never any increase in the total protein content. On the contrary, the peptones were broken down, and converted into crystalline decomposition products. This hydrolysis was effected by the enzyme erepsin, a body quite distinct from trypsin, in that it has no action upon native proteins but only upon their decomposition products. According to Cohnheim, the proteins

[1] Cathcart, *Journ. Physiol.*, 32, p. 299, 1905.

[2] Cohnheim, *Zeit. f. physiol. Chem.*, 33, p. 451, 1901; and 35, p. 134, 1902.

[3] Hofmeister, *ibid.*, 6, p. 69; and *Arch. f. exp. Path.*, 19, p. 8, 1885.

of horses' plasma, of ascitic fluid, of muscle, and vitellin and globin are not acted upon by erepsin even during several weeks. Primary proteoses are not appreciably attacked in thirty-six hours, though they undergo gradual hydrolysis in the course of days. Deutero-proteose B (prepared by Pick's method) is split up in nineteen hours to the stage at which it no longer gives the biuret test, and peptone (prepared by the prolonged peptic digestion of muscle) in as little as two hours. The protamine clupein sulphate is quickly hydrolysed, and, contrary to expectation, casein is likewise decomposed somewhat readily.

Erepsin has been shown by Kutscher and Seemann[1] to be present in succus entericus, and doubtless it is of importance in assisting the pancreatic trypsin to break up the proteoses and peptones in the gut. But Cohnheim thinks that in all probability its more important seat of action is within the cells of the intestinal mucous membrane, not outside them. Whether the intra- and extra-cellular erepsins have identically the same action upon proteoses and peptones has not been determined, but there is no reason to suppose that the two enzymes are different bodies. They both act best in fairly alkaline solution, and have little or no digestive power in an acid one, and both give similar decomposition products, so far as is known. It is suggested by Abderhalden and Teruuchi[2] that the term "erepsin," being comparable to "trypsin" and "pepsin," ought to be confined to the proteolytic enzyme of the succus entericus, and not applied to the corresponding endoenzyme. If this suggestion be adopted, the endoenzyme could be spoken of as "endoerepsin," or "ereptase."

In the light of our present knowledge, it might be predicted that most intracellular enzymes found in one tissue or organ of the body would, in all probability, be found in greater or less degree in other tissues. This is true of erepsin, as in 1903[3] I showed that the pancreas contains an ereptic enzyme which is quite distinct from trypsin, and in 1904[4] that erepsin is probably

[1] Kutscher and Seemann, *Zeit. f. physiol. Chem.*, 35, p. 432, 1902.
[2] Abderhalden and Teruuchi, *ibid.*, 49, p. 1, 1906.
[3] Vernon, *Journ. Physiol.*, 30, p. 330, 1903.
[4] *Ibid.*, 32, p. 33, 1904.

present in all animal tissues. In order to obtain comparable results, the relative amounts of enzyme in the tissue extracts were determined quantitatively by a colorimetric method dependent on the biuret test. I found that the time required to split up any given percentage of a standard solution of Witte's peptone to the non-biuret-test-giving stage varied inversely as the quantity of enzyme present. For instance, in one experiment 8, 4, 2, and 1 parts of enzyme split up 20 per cent. of the peptone in ·7, 1·4, 2·8, and 6·6 hours respectively. The same amounts of enzyme split up 30 per cent. in 1·9, 3·4, 6·8, and 15·7 hours respectively, and 40 per cent. in 4·2, 7·4, 13·8, and 33·8 hours. In the majority of cases the time required by a tissue extract to split up 20 per cent. of a 2·5 per cent. peptone solution was determined, when acting at 38° C. in presence of ·1 per cent. Na_2CO_3 and toluol. The relative amounts of erepsin found to be present in the various tissues of the cat were the following:[1]—

Tissue.	Ereptic Value.	Tissue.	Ereptic Value.
Duodenal muc. memb.	27·7	Submaxillary gland	5·3
Jejunal „	18·2	Thyroid gland	4·3
Ileal „	14·4	Suprarenal gland	2·5
Large intest. „	5·8	Cardiac muscle	1·6
Gastric „	3·9	Brain	1·2
Kidney	14·3	Ovary	1·0
Spleen	7·6	Skeletal muscle	·8
Lung	6·9	Blood	·3
Pancreas	6·4	Serum	·1
Liver	5·0		

It will be seen that the duodenal mucous membrane is richest of all in erepsin, and that there is a steady diminution in enzyme on passing down the gut. Of the other tissues, the kidney comes easily first, being twice as rich in erepsin as any other organ. Blood and serum contain only a very small amount of the ferment.

The dependence of the hydrolytic power of erepsin upon an alkaline medium is shown by the following data, obtained with cat's kidney extract:—

[1] Vernon, *ibid.*; and *Journ Physiol.*, 33, p. 81, 1905.

Extract acting in presence of	Relative Digestive Power.
Water only	3·7
·1 per cent. Na_2CO_3 . . .	13·3
·2 ,, Na_2CO_3 . . .	22·2
·4 ,, Na_2CO_3 . . .	28·2
·05 ,, Acetic Acid . . .	·4
·1 ,, Acetic Acid . . .	·17

It will be seen that the rate of hydrolysis is greater and greater the more alkaline the solution. However, this is true only if the time of hydrolysis of 20 per cent. of the peptone be taken as a measure of digestive power. The strong alkali destroys the enzyme, so that 40 per cent. of the peptone was more quickly hydrolysed in presence of ·1 per cent. Na_2CO_3 than in ·4 per cent. Na_2CO_3.

The question arises as to whether the ereptic enzyme in the various tissues is in all cases the same body. The evidence, though not conclusive, points distinctly to the existence of different enzymes in different tissues. I found that various partially hydrolysed peptones were further split up at very different relative rates by the different extracts. Again, the different extracts were differently affected by the alkalinity and acidity of the medium in which they were acting. Those of cat's tissues, for instance, were in some cases found to be 60 or 70 times more active in presence of ·1 per cent. Na_2CO_3 than in ·1 per cent. acetic acid, whilst in others they were only 12 times more active. The erepsin in pigeon's tissues was much less retarded by acid, for it was only three to five times less active in ·1 per cent. acetic acid than in ·1 per cent. Na_2CO_3. It seems probable, therefore, that the tissues not only contain somewhat different erepsins, but different relative amounts of acid-acting ferment and of alkali-acting ferment. But it is to be remembered that the tissue extracts undoubtedly contain proteins and other substances which may retard or accelerate the action of the enzymes, and until these enzymes can be prepared free from such impurities, it is best not to speak dogmatically.

The differences between endoenzymes and trypsin is well shown by their action upon polypeptides. Abderhalden, in

conjunction with Emil Fischer,[1] Teruuchi,[2] Rona,[3] and Hunter,[4] found that the press juice of liver, kidney, and muscle, hydrolyses certain polypeptides such as dl-leucyl-glycin, glycyl-dl-alanin and glycyl-glycin which trypsin has no action upon. Kidney juice was the most active; liver juice somewhat less so, and muscle juice very much less so. The first two of the three polypeptides mentioned are racemic bodies, and in each case only one of the two stereoisomers was attacked by the endoenzymes. The active amino acid liberated was that which is present in native proteins, viz., l-leucin in the one case and

Polypeptide.	Ox or Dog's Liver Juice.	Dog's Kidney Juice.	Ox or Dog's Muscle Juice.	Trypsin.
Dl-leucyl-glycyl-glycin .	+	...	...	+
Dl-alanyl-glycyl-glycin .	+	...	...	+
Glycyl-l-tyrosin . .	+	...	+	+
Dl-leucyl-glycin . .	+	+	+	−
Glycyl-dl-alanin . .	+	+	+	−
Glycyl-glycin . . .	+	+	+	−
Leucyl-leucin . . .	−	...	...	−

d-alanin in the other. Other peptides, such as leucyl-leucin, resist the attack of endoenzymes as well as of trypsin, whilst the first three peptides recorded in the table are split up by both endoenzymes and trypsin. They are not attacked by pepsin, however, so by means of these synthetic polypeptides we are able to differentiate sharply between the three proteolytic enzymes. If the proteases, endoerepsins and other proteolytic endoenzymes could be separated from one another, it would probably be found that they too exerted a differential action upon certain of the polypeptides: but at present we are ignorant as to the precise endoenzyme responsible for the peptide hydrolyses. No acid or alkali was added to the digests, but in that the tissue juices have a considerable natural acidity, it might be supposed that the β-protease enzyme was chiefly responsible. However, Abderhalden and Teruuchi found that fresh succus entericus, which has a con-

[1] Fischer and Abderhalden, *Zeit. f. physiol. Chem.*, 46, p. 52, 1905.
[2] Abderhalden and Teruuchi, *ibid.*, 47, p. 466; and 49, p. 1, 1906.
[3] Abderhalden and Rona, *ibid.*, 49, p. 31.
[4] Abderhalden and Hunter, *ibid.*, 48, p. 537.

siderable natural alkalinity, could split up glycyl-glycin just like the tissue juices, and also we have seen that these juices can hydrolyse peptones slowly in faintly acid solution, so it is possible that the peptide hydrolysis is due to endoerepsin, or to this enzyme and β-protease acting concurrently.

Other evidence differentiating trypsin and endoenzymes is yielded by quantitative studies of the course of protein hydrolysis. Abderhalden, working in conjunction with Reinbold[1] and Vögtlin,[2] found that certain of the amino acid constituents of proteins, such as tyrosin and tryptophan, were quickly split off from the protein complex by the action of activated pancreatic juice, and could be recovered quantitatively. Other constituents, such as glutamic acid, aspartic acid, leucin, valin, and alanin, were only gradually and often not completely liberated, whilst others again, such as prolin and phenyl-alanin, were scarcely liberated at all. As already mentioned, Dakin isolated both of these latter bodies from an acid kidney autolysis, and Cathcart isolated them from a digest of blood serum with α-protease in alkaline solution. Hence the tissue endoenzymes carry the protein hydrolysis to a further stage than trypsin. But it is not yet proved that they can carry it to absolute completion in the same way that inorganic catalysts can do. Abderhalden and Prym[3] hydrolysed the proteins of liver pulp by boiling with hydrochloric acid, and from the mixture they isolated, by the ester method, 42·5 to 45·8 gms. of mono-amino acids for each 100 gms. of liver protein taken. Some of the liver pulp was incubated in presence of water and toluol for ten to fifty days, and the following amounts of mono-amino acids (calculated for 100 gms. of liver protein) were isolated from the autolysis :—

Duration of Autolysis.	10 days.	20 days.	30 days.	40 days.	50 days.
	Gms.	Gms.	Gms.	Gms.	Gms.
Mono-amino acids . .	1·85	5·5	10·1	20·2	29·1

Assuming that the ester method yields roughly quantitative

[1] Abderhalden and Reinbold, *Zeit. f. physiol. Chem.*, 44, p. 284, 1905 ; and 46, p. 159, 1905.

[2] Abderhalden and Vögtlin, *ibid.*, 53, p. 315, 1907.

[3] Abderhalden and Prym, *ibid.*, 53, p. 320, 1907.

results, we see that even after fifty days' autolysis only about two-thirds of the total mono-amino acids were liberated. Still the autolysis seemed to be progressing at a steady rate at the time it was stopped, and it is very probable that if only it had been continued for another twenty or thirty days, it would have attained the completeness of the acid hydrolysis.

An interesting and important fact noted by Abderhalden and Prym in their autolyses was the early disappearance of the biuret test. It was distinct for the first day or two, but had disappeared on the fourth day. At this time extremely little of the protein had been hydrolysed to the mono-amino acid stage, and hence presumably the protein decomposition products consisted chiefly of various unknown dipeptide amino acid combinations which were too simple in constitution to yield the biuret test. Thus the work of Schiff[1] has shown that in order to give this test a substance must contain two —CO.NH— groupings, joined indirectly or directly. So tripeptides give it, but dipeptides—unless they are amides—do not.

Arginase and similar Endoenzymes.—The third class of proteolytic endoenzymes is at present even less clearly defined than the two classes so far described. It includes enzymes which act upon individual amino acids, and split them up into still smaller molecules. The best known member of this class is arginase. This enzyme was discovered by Kossel and Dakin[2] in 1904, and it has the special property of hydrolysing arginin to urea and ornithin (diamino-valerianic acid). Kossel and Dakin point out that this hydrolysis differs from those effected by other "imidolytic" ferments such as trypsin and erepsin, for they attack the —CO—NH—C— groupings of proteins and polypeptides, and split them into —COOH and NH_2—C— groupings. Arginase, on the other hand, splits off urea according to the equation:

$$NH_2-\overset{\overset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{CH}-COOH+H_2O$$

$$=NH_2-CO-NH_2+NH_2-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{CH}-COOH$$

[1] Schiff, *Ber.*, 29, p. 298, 1896; *Ann. Chem. Pharm.*, 299, p. 236, 1897.

[2] Kossel and Dakin, *Zeit. f. physiol. Chem.*, 41, p. 321, 1904.

Arginase was found to be present in largest quantity in the liver. For instance, 25 gms. of minced liver, placed in an incubator with 1000 c.c. of water and toluol, completely hydrolysed 5·0 gms. of arginin in six hours. In another experiment, 25 gms. of liver hydrolysed 2·7 gms. out of 3·2 gms. of arginin in ten minutes. No other organ proved anything like so active. The best of them was the kidney, and it was found that 25 gms. of minced calf's kidney, when allowed to act upon 2·2 gms. of arginin for three days, decomposed 1·14 gms. of it. Slightly less active were thymus and lymph glands. Intestinal mucous membrane was considerably less active, and muscle still less. Blood contained only traces of arginase, and spleen, suprarenal gland, and pancreatic juice apparently none whatever. These conclusions are in agreement with the observations of various investigators upon the products of tissue autolysis. Thus Kutcher and Seemann[1] found no arginin in autolyses of the thymus and of intestinal mucous membrane, and Dakin[2] found none in those of the kidney. On the other hand, Leathes[3] obtained a good yield of arginin from a spleen autolysis which had been digesting two to four months.

Dakin[4] concludes that arginase is a specific enzyme adapted for the exclusive hydrolysis of dextro-rotatory arginin, or of substances containing the dextro-arginin grouping. He finds that it has no action upon guanidin, creatin, or creatinin, or on protamines or other proteins: but it acts upon the arginin complex present in certain protones, and liberates urea therefrom.

The definite localisation of arginase in particular organs proves that the enzyme is quite distinct from proteases and erepsins, for these ferments are probably present in every tissue of the body. There is reason to think that other proteolytic enzymes exist which are similarly confined to particular organs, and to particular purposes, but in their case the evidence is not very complete or convincing. Lang[5] made a number of

[1] Kutscher and Seemann, *Zeit. f. physiol. Chem.*, 34, p. 114, 1901; and 35, p. 432, 1902.

[2] Dakin, *Journ. Physiol.*, 30, p. 84, 1903.

[3] Leathes, *ibid.*, 28, p. 360, 1902.

[4] Dakin, *Journ. Biol. Chem.*, 3, p. 435, 1907.

[5] Lang, *Hofmeister's Beitr.*, 5, p. 321, 1904.

determinations of the power possessed by various tissues to split off ammonia from certain amino acids and other nitrogenous bodies. The method consisted in mixing up the minced tissue thoroughly with ·9 per cent. NaCl, toluol and the amino acid, and allowing it to incubate for two to thirty-two days. It was then acidified, and 5 to 8 per cent. tannin solution added. The filtrate was distilled with magnesia in a vacuum at 40° to 45° (Nencki and Zaleski's method),[1] and the ammonia which came off collected and estimated. Lang found that from glycin minced intestinal mucous membrane and pancreas separated a considerable amount of ammonia; kidney, suprarenal gland, testis, and liver separated a moderate amount; and spleen and lymph glands none at all. From glucosamin, kidney and suprarenal gland separated the most ammonia; liver, intestine, testis, and spleen a moderate amount; muscle very little; and pancreas none at all. Other experiments were made with tyrosin, leucin, and cystin, and every one of these substances was apparently hydrolysed to some extent by one or other of the tissues experimented with. Unfortunately the results, though of considerable interest, cannot be accepted unreservedly. Many of them are based upon a single experiment only, and are sometimes self-contradictory. For instance, liver digested with glucosamin for five days yielded 62 mg. of NH_3, as against 45 mg. from liver digesting without glucosamin. After digesting nine days, however, liver *plus* glucosamin gave only 53 mg. of NH_3, but liver alone, 92 mg. Again, in some cases liver and kidney tissue to which glycin had been added yielded less NH_3 than without any addition, and indeed Jacoby[2] has stated that minced liver is quite incapable of hydrolysing glycin. Still there can be no doubt that the tissues contain enzymes which have the power of liberating ammonia from some or other amino acids and amides, and that certain structures, such as the liver, are much more active than others such as muscle.

Previous to Lang, the ammonia-liberating power of liver endoenzymes was studied in considerable detail by Jacoby.[3] He found that if ground-up liver tissue were kept at 38° with an

[1] Nencki and Zaleski, *Zeit. f. physiol. Chem.*, 33, p. 193, 1901.

[2] Jacoby, *ibid.*, 30, p. 149, 1900. [3] *Ibid.*

equal volume of water and toluol, the amount of ammonia set free on boiling with magnesia, or the "amide" nitrogen, steadily increased. For instance, in one experiment it was ·0013 gm. after one day's autolysis, ·0035 gm. after two to five days', ·0047 gm. after eleven to fifteen days', and ·0067 gm. after twenty days' autolysis. In another experiment, he found that after fourteen days' digestion the nitrogen still present in the form of protein had fallen from 94 per cent. of the whole down to 27 per cent., whilst the amide nitrogen had increased from 1·1 per cent. to 5·6 per cent. In another experiment, after eighteen days' digestion, it had increased from ·4 per cent. to 8·4 per cent. Other observers have obtained similar results with other tissues. Dakin[1] allowed kidney juice to digest itself under antiseptic conditions, and he found that the ammonia increased slowly and steadily for about two months.

The only amide known to be present in the protein molecule from which the amide nitrogen could be derived is urea, and this can account for only a small fraction of the amide nitrogen obtainable. But in the light of Lang's experiments, we may assume that part of it is derived from certain of the amino acids, though we cannot definitely say which of them.

Hausmann,[2] Gümbel,[3] Osborne and Harris,[4] and others, have shown that if proteins be dissociated by boiling with HCl, they yield a definite proportion of their nitrogen in the form of ammonia which can be driven off by subsequent distillation with magnesia. Casein yields 10 to 13 per cent. of its total nitrogen in this way, edestin 12 per cent., serum albumin 7 per cent., but gelatin only 1·6 per cent. Hirschler,[5] Stadelmann,[6] and others, showed that trypsin has the power of liberating ammonia from the protein molecule, whilst Zunz,[7] and Dzierzgowski and Salaskin,[8] found that pepsin likewise has this

[1] Dakin, *Journ. Physiol.*, 30, p. 84, 1904.
[2] Hausmann, *Zeit. f. physiol. Chem.*, 27, p. 95, 1899; and 29, p. 136, 1900.
[3] Gümbel, *Hofmeister's Beitr.*, 5, p. 297, 1904.
[4] Osborne and Harris, *Journ. Amer. Chem. Soc.*, 25, p. 323, 1903.
[5] Hirschler, *Zeit. f. physiol. Chem.*, 10, p. 302, 1886.
[6] Stadelmann, *Zeit. f. Biol.*, 24, p. 261, 1888.
[7] Zunz, *Zeit. f. physiol. Chem.*, 28, p. 151, 1899.
[8] Dzierzgowski and Salaskin, *Centralb. f. Physiol.*, 15, p. 249, 1902.

power. The latter investigators found that peptic digestion of egg albumin for eighteen days split off 2·6 per cent. of the total nitrogen in the form of ammonia, whilst peptic digestion of casein for ten days split off 3·5 per cent. Zunz found that peptic digestion of serum albumin for fifteen days split off 2·1 per cent., and of casein for fifteen days, 3·8 per cent. These amounts are only a half to a third those observed by Jacoby for liver autolysis, and by Hausmann and others for acid hydrolysis.

The amino acids and amides hydrolysed by endoenzymes are probably to some extent different from those hydrolysed by acids, for Jacoby found that if liver juice were first allowed to digest itself, and were then boiled with HCl, it yielded considerably more ammonia than in the absence of an initial autolysis. The data obtained in one experiment were :—

	Without Autolysis.	After Autolysis.
	Per cent.	Per cent.
Amide Nitrogen, directly separable . .	2·6	9·4
,, separable by acids . .	8·7	6·3
Total Amide Nitrogen . .	11·3	15·7

In another experiment the amide nitrogen amounted to 9·6 per cent. without autolysis, and 15·5 per cent. with it.

The Formation and Hydrolysis of Urea by Enzymes.—Charles Richet[1] in 1893 found that dog's liver, freshly removed from the body and kept at 39°, showed an increase in its content of urea. Later on[2] he found that an aqueous filtered extract of liver tissue, digesting in presence of NaF, likewise possessed this urea forming power, and even that the precipitate thrown down by the addition of alcohol to the extract possessed it. Gottlieb[3] observed urea formation in liver pulp kept at 40° under aseptic conditions, whilst Schwarz[4] found that a digest of liver pulp showed an increase in its nitrogenous ether-alcohol extractives.

[1] Richet, *Comptes Rendus*, 118, p. 1125, 1893.
[2] Richet, *C. R. Soc. Biol.*, 1894, pp. 368 and 525.
[3] Gottlieb, *Münch. Med. Wochenschr.*, 1895.
[4] Schwarz, *Arch. f. exp. Path.*, 41, p. 60, 1898.

These extractives were not ammonia, and so were presumably urea.

There can be no doubt, therefore, as to the formation of urea in liver pulp and liver extracts, but the amount produced is small, and, as Kossel and Dakin suggest, it may be formed by the arginase hydrolysing the arginin formed by autolysis. There can be no doubt that some of it arises in this way, but probably not all. O. Loewi[1] found that the liver enzymes could convert glycin and leucin into a nitrogenous body which, though not actually urea, behaved like urea in respect of its solubility in alcohol, its easily separable nitrogen, and in other ways. Not only could liver extracts effect this change, but also an aqueous extract of the precipitate thrown down by 97 per cent. alcohol. The enzyme had no influence upon alanin or ammonium salts, however. Previous to Loewi, Chassevant and Richet[2] observed that liver extracts could convert alkaline urates into urea, but Spitzer[3] failed to observe any urea formation when liver pulp and extracts were allowed to act either upon leucin, urates, or ammonium salts. The origin of urea from any other source but arginin is therefore unproved, and the subject requires re-investigation.

The hydrolysis of urea by enzyme action is less open to question than its formation. Jacoby[4] found that if liver juice were allowed to act upon urea in presence of toluol for thirty-six hours, more than twice the amount of ammonia was set free on subsequent boiling with magnesia, as in the control experiment in which no urea was added. Lang[5] found that liver pulp split off a small quantity of ammonia from urea, whilst minced pancreas split off a larger amount.

The existence of a urea-splitting enzyme in fermenting urine was demonstrated by Musculus[6] as long ago as 1874. This *urease*, as he called it, is formed by the activity of the *Micrococcus ureae*, and it seems to be entirely an endoenzyme, for Sheridan

[1] O. Loewi, *Zeit. f. physiol. Chem.*, 25, p. 511, 1898.
[2] Chassevant and Richet, *Comptes Rendus Soc. Biol.*, 1897, p. 793.
[3] Spitzer, *Pflüger's Arch.*, 70, p. 60, 1898.
[4] Jacoby, *Zeit. f. physiol. Chem.*, 30, p. 149, 1900.
[5] Lang, *Hofmeister's Beitr.*, 5, p. 321, 1904.
[6] Musculus, *Comptes Rendus*, 78, p. 132, 1874; 82, p. 334, 1876.

Lea[1] found that fermenting urine, when freed from the Micrococcus by adequate filtration, has no power of splitting up urea. Again, Leube[2] did not find any soluble ferment in filtrates from pure cultures of the organism. Musculus precipitated alkaline pathological urine with alcohol, and found that an aqueous extract of the dried precipitate was very active. Lea precipitated fermenting urine in the same way, and found that the dried alcohol precipitate, or an aqueous extract of it, when mixed with 2 per cent. urea solution and kept at 38°, turned it alkaline and liberated ammonia in a few minutes. The destruction of the micro-organisms by the alcohol precipitation apparently sets free the urease, and renders it capable of extraction. However, Beijerinck[3] was unable to extract it from dead micrococci, and he considers that it is firmly bound up in the cells. Moll[4] found that even small quantities of antiseptics, such as toluol and chloroform, render it inactive, but that sodium fluoride is not so harmful.

This urea-splitting enzyme is not by any means confined to the Micrococcus ureae. Miquel[5] demonstrated its presence in a number of other Micrococci and Bacilli, and in a Sarcina. He cultivated no less than 14 different species of micro-organisms in peptone solutions containing ·2 to ·3 per cent. of ammonium carbonate, and he found that after some days these solutions contained a good deal of soluble enzyme which had been excreted by the micro-organisms. But he gives no details of his experiments, and in the face of the contrary assertions of the other investigators, we cannot accept his statements as proven.

The urease of micro-organisms acts best in an alkaline medium. The urease of the liver and other tissues mentioned above was allowed to act at the natural acidity of the tissue juices. Hence it is probably a different enzyme. But until it is to some extent isolated from other endoenzymes and tissue constituents, and obtained in a state of greater concentration, we cannot decide the point definitely.

[1] Lea, *Journ. Physiol.*, 6, p. 136, 1885.
[2] Leube, *Virchow's Arch.*, 100, p. 540, 1885.
[3] Beijerinck, *Centralb. f. Bakt.* (2) 7, p. 33, 1901.
[4] Moll, *Hofmeister's Beitr.*, 2, p. 244, 1900.
[5] Miquel, *Comptes Rendus*, 111, p. 397, 1890.

Summary.—We have seen that the proteolytic endoenzymes of mammalian tissues, so far as they are known, form a graduated series, each succeeding member of which acts more especially upon the digestion products produced by a preceding member.

In addition to the acid- and alkali-acting proteases and erepsins, and to arginase, there are probably other distinct enzymes concerned in the hydrolysis of individual amino acids, but at present we have no exact knowledge of them. It is possible that urease is identical with the enzyme or enzymes which split off ammonia from the individual amino acids, and the fact that the Micrococcus ureæ has been found by Van Tieghem [1] to decompose hippuric acid into glycin and benzoic acid, lends support to this view. But it seems more probable, judging from what is known as to the close correlation between individual sugars and sucroclastic enzymes,[2] and of the capacity of the tissues to elaborate innumerable protein molecules, such as antitoxins, antiferments, lysins, agglutinins, precipitins, etc., each of a different configuration and with a special function, that these tissues are built up of a very large number of different endoenzymes, each specially adapted for some particular and closely defined purpose.

[1] Van Tieghem, *Comptes Rendus*, 58, p. 533, 1864.

[2] See Lect. VII., p. 170.

LECTURE II

PROTEOLYTIC ENDOENZYMES—*continued*

Action of pepsin, trypsin, and proteases on nucleoproteins. Presence of nuclease in all tissues. Irregular distribution of adenase, guanase, and xantho-oxidase. Uricolytic enzyme and its action. Relation between endoenzymes and functional capacity of tissues, as shown by effects of development, disease, food, and hibernation. Presence of proteolytic endoenzymes in all organisms, and their reaction to acids and alkalis. Plant enzymes, both peptic and ereptic.

THE nucleoproteins, like the proteins, seem to need a whole group of endoenzymes peculiar to themselves to bring about the later stages of their decomposition. The earlier stages are readily induced by the enzymes of the alimentary canal. Pepsin and hydrochloric acid quickly split up nucleoproteins into a protein fraction, which undergoes further hydrolysis into proteoses and peptones, and leave an insoluble precipitate of nuclein. Milroy[1] and Umber[2] have shown that some of this nuclein is split up further to the nucleic acid stage, but probably most of this hydrolysis is effected in the ordinary course of digestion by the pancreatic trypsin. Apparently trypsin has no further action upon nucleic acid, and the succeeding stage of hydrolysis is effected by the erepsin of the intestinal juice; but our information upon this point is very vague. The action of intracellular enzymes upon nucleoproteins is much more complete. No special observations on the earlier stages of hydrolysis have been made, but there is no doubt that the tissue proteases, probably those acting in presence of alkalis, as well as

[1] Milroy, *Zeit. f. physiol. Chem.*, 22, p. 307, 1896.
[2] Umber, *Zeit. f. klin. Med.*, 43, 1901.

those acting with acids, readily split up nucleoproteins to the nucleic acid stage. The further hydrolysis is effected by special nuclease, guanase, adenase, and other enzymes.

The existence of intracellular nucleoprotein-hydrolysing enzymes was first pointed out by Salomon,[1] who in 1878 showed that muscle, pancreas, and liver contain an enzyme which can liberate xanthin bases from the tissues. These xanthin bases were presumably derived from the nucleins present. Salkowski and his pupils showed that the enzyme responsible for this hydrolysis was present in filtered aqueous extracts of glands and of yeast cells. The enzyme is distinct from trypsin, in that its action is retarded by alkalis. Also Iwanoff[2] found that the "nuclease," as he termed it, which is present in Fungi such as *Aspergillus niger* and *Penicillium glaucum*, rapidly split up nucleic acid into phosphoric acid and xanthin bases, but was incapable of liquefying gelatin. Jones[3] showed that the nucleoprotein of thymus, freed from all other glandular constituents, retained an enzyme which was capable of hydrolysing it to phosphoric acid and xanthin bases, and that this hydrolysis was quickly stopped by alkalis. Again, Sachs[4] tested the action of nuclease upon the sodium salt of the α-nucleic acid obtained from the thymus gland. A 4 per cent. solution of this body forms a firm jelly at room temperature, and nucleases liquefy this jelly and convert it first into the soluble β-nucleate, and then liberate phosphoric acid and xanthin bases; but trypsin is without action upon it. Recently prepared pancreatic extracts and pancreas press juice, though possessing little or no tryptic power owing to the trypsin being in the zymogen form, were able to split up the nucleate, as they contained some nuclease. If the extracts were kept, however, the trypsin was liberated and soon destroyed the nuclease, just as the writer[5] found that the trypsin of pancreatic extracts gradually destroys the endoerepsin present.

Nuclease has been found by Jones, Partridge, and Winternitz[6]

[1] Salomon, *Zeit. f. physiol. Chem.*, 2, p. 65, 1878.

[2] Iwanoff, *ibid.*, 39, p. 31, 1903. [3] Jones, *ibid.*, 41, p. 101, 1904.

[4] Sachs, *ibid.*, 46, p. 337, 1905.

[5] Vernon, *Journ. Physiol.*, 30, p. 341, 1903.

[6] Jones and Partridge, *Zeit. f. physiol. Chem.*, 42, p. 343, 1904; Jones and Winternitz, *ibid.*, 44, p. 1, 1905.

in many other organs, such as the spleen, suprarenal gland, and liver, so it is probably present in all glands. In that all tissues contain nucleoproteins, one would expect nuclease to be universally present. But Sachs, though he found it in the pancreas, thymus, and kidney, failed to trace it in muscle. Doubtless muscle contains much less of the enzyme than glandular tissues, as it is so much poorer in nucleoproteins, but there can be little doubt that it would be found if the methods of detection were sufficiently delicate.

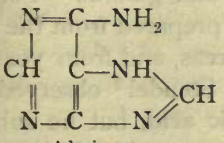
It seems probable that nuclease is an enzyme *sui generis*, and not to be confounded with any of the proteolytic endoenzymes mentioned in the previous lecture. It is distinct from β-protease, for Arinkin[1] found that the addition of ·3 per cent. or less of various mineral and organic acids, though it accelerated the autolysis of the protein constituents of liver pulp, retarded the formation of purin bases. Lactic acid retarded the nucleic acid hydrolysis least of all, and in one experiment in which ·06 per cent. of the acid was added, it accelerated it.

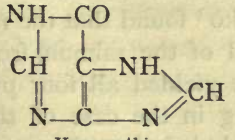
The non-identity of nuclease and erepsin seems to be proved by the fact that nuclease acts best in a neutral medium or at the natural acidity of tissue extracts,[2] whilst erepsin acts best in alkaline solution. Nakayama[3] found that ground-up extracts of intestinal mucous membrane, when acting at the natural acidity of the extract, decomposed sodium α-nucleate, and he attributed this action to erepsin; but there can be no doubt that such an extract contained nuclease, and the whole of the observed action may have been effected by this enzyme.

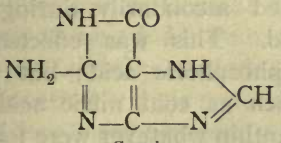
Before discussing the further changes undergone by the purin bases liberated from nucleic acid, it will be well to point out briefly the chemical relationships of these bodies to one another and to uric acid. As we see from the structural formulæ, adenin is an amino-hypoxanthin, and it can react with water to form ammonia and hypoxanthin. Similarly, guanin is amino-xanthin, which can react to form ammonia and xanthin. Xanthin contains one oxygen atom less than uric acid. Uric acid, when acted on by a mild oxidising agent such as potassium permanganate, is converted into allantoin

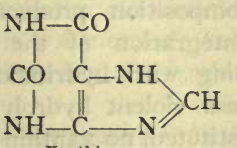
[1] Arinkin, *Zeit. f. physiol. Chem.*, 53, p. 192, 1907.
[2] Kikkōji, *ibid.*, 51, p. 201, 1907. [3] Nakayama. *ibid.*, 41, p. 348, 1904.

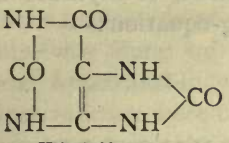
and carbon dioxide, whilst a stronger oxidising agent, such as cold nitric acid, converts it into alloxan and urea. By other

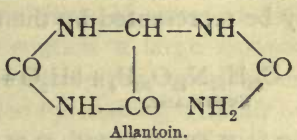

Adenin.


Hypoxanthin.


Guanin.


Xanthin.


Uric Acid.


Allantoin.

treatment this alloxan can be split up into carbon dioxide, oxalic acid and another molecule of urea.[1]

The nature and number of the molecules of purin bases present in a molecule of nucleic acid is a matter of considerable doubt. Bang[2] prepared a nucleic acid from the pancreas which contained only guanin, and the structural formula he gives for the acid contains four guanin molecules. He represents its hydrolysis by the following equation:—

$$\underset{\text{Nucleic Acid.}}{C_{44}H_{66}N_{20}P_4O_{34}} + 10H_2O$$

$$= \underset{\text{Guanin.}}{4C_5H_5N_5O} + \underset{\text{Pentose.}}{3C_5H_{10}O_5} + \underset{\text{Glycerin.}}{3C_3H_8O_3} + \underset{\text{Phosphoric Acid.}}{4H_3PO_4}$$

As a rule, the nucleic acids prepared from various tissues yield adenin as well as guanin, but there is no good proof that the preparations are not mixtures of two nucleic acids,

[1] *Cf.* article by Macleod in Hill's *Recent Advances in Bio-Chemistry*, p. 388, 1906.

[2] Bang, *Zeit. f. physiol. Chem.*, 31, p. 411, 1900.

one containing only guanin, and the other only adenin. Some analyses of nucleic acids show the presence of xanthin and hypoxanthin, as well as of adenin and guanin. For instance, Inoko[1] found that the nucleic acids prepared from the spermatic fluid of the salmon, from the pancreas, and from yeast, in each case yielded all four purin bases. Steudel[2] observed the same thing in the case of thymus nucleic acid, but he subsequently concluded that the xanthin and hypoxanthin isolated from the decomposition products are formed secondarily during the disintegration of the nucleic acid. This was effected by boiling with hydriodic acid and phosphoric acid; but when a less violent hydrolytic agent such as cold nitric acid was substituted, no xanthin and hypoxanthin whatever were formed. Steudel[3] thinks that the decomposition of thymus nucleic acid may be represented by the following equation:—

$$\underset{\text{Nucleic Acid.}}{C_{43}H_{57}N_{15}O_{30}P_4} + 8H_2O + O_2$$

$$= \underset{\text{Guanin.}}{C_5H_5N_5O} + \underset{\text{Adenin.}}{C_5H_5N_5} + \underset{\text{Thymin.}}{C_5H_6N_2O_2} + \underset{\text{Cytosin.}}{C_4H_5N_3O} + 4\underset{\text{Hexose.}}{C_6H_{12}O_6} + 4\underset{\text{Metaphosphoric Acid.}}{HPO_3}$$

The formula given for the nucleic acid differs somewhat from that derived by Miescher, Schmiedeberg, and Kostytschew from their analyses, but Steudel shows that it stands in fair agreement with their quantitative results. Also, from the hydrolytic products of a known weight of the acid, Steudel isolated approximately the quantities of guanin, adenin, and thymin required by this equation; but the cytosin obtained was too low in amount, as some of it underwent a secondary oxidation to uracil. No pentose was isolated by him, though we saw that the pancreas nucleic acid examined by Bang contained the whole of its carbohydrate in this form. Burian[4] gives a tentative formula

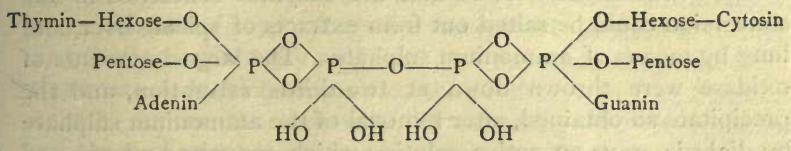
[1] Inoko, *Zeit. f. physiol. Chem.*, 18, p. 540, 1893; see also, G. Mann's *Chemistry of the Proteids*, London, 1906, p. 440.

[2] Steudel, *Zeit. f. physiol. Chem.*, 42, p. 165, 1904.

[3] *Ibid.*, 53, p. 14; also, *Centralb. f. Physiol.*, 21, p. 472, 1907.

[4] Burian, quoted from Loeb, *University of California Publications in Physiology*, 3, p. 62, 1907.

for nucleic acid with two hexose groups and two pentose groups:—



These several formulæ show how greatly the nucleic acids of various tissues differ from one another in constitution; but we may provisionally conclude that none of them contain xanthin or hypoxanthin in their molecules.

Adenase and Guanase.—The action of intracellular enzymes upon the purin bases was first studied by Horbaczewski[1] in 1891. Minced spleen, kept with nine parts of water at 50° for eight hours, was found by him to contain a large amount of xanthin and hypoxanthin, whereas fresh spleen contained none. If the spleen mixture were oxygenated by shaking with air or by adding hydrogen peroxide or blood to it, it was found to contain uric acid instead of hypoxanthin and xanthin. Horbaczewski's experiments were carried out in the absence of antiseptics, so a certain amount of putrefaction ensued. The reaction was not dependent on putrefaction, however, as Spitzer[2] obtained the same result in the presence of chloroform or thymol. Also, Spitzer found that if xanthin and hypoxanthin were added to extract of spleen or liver which was kept at 50° with a continuous stream of air bubbling through, they were directly converted into uric acid. In one experiment, at least 90 per cent. of the xanthin and hypoxanthin added was converted. On the other hand, extracts of kidney, pancreas, and thymus, acting under similar conditions, yielded no uric acid whatever. Spitzer found that liver and spleen extracts likewise possessed the power of converting adenin and guanin into uric acid, though the conversion was not effected so readily as that of xanthin and hypoxanthin. That is to say, the enzymes which split off the amino grouping from the guanin and adenin, and so converted them

[1] Horbaczewski, *Sitzungsber. d. Wiener Akad. d. Wiss. Mathemnaturw. Classe*, 100, 1891.

[2] Spitzer, *Pflüger's Arch.*, 76, p. 192, 1899.

into xanthin and hypoxanthin respectively, were not so active as the oxidising enzyme which converted these bodies into uric acid. Schittenhelm [1] found that the enzymes concerned in this conversion could be salted out from extracts of spleen, liver, and lung by means of ammonium sulphate. The largest amounts of oxidase were thrown down at two-thirds saturation, and the precipitate so obtained, after removal of the ammonium sulphate by dialysis, gave an active solution which converted adenin and guanin almost quantitatively into uric acid. At least this was the case if air were bubbled through the solution. An experiment made with guanin showed that in absence of air only the xanthin stage was reached.

Arguing from comparisons of the activities possessed by various tissue extracts, Jones, Austrian, and Winternitz [2] conclude that in the conversion of the amino purins into uric acid three distinct enzymes are concerned. A guanase converts guanin into xanthin; an adenase converts adenin into hypoxanthin, whilst a xantho-oxidase oxidises these two oxypurins into uric acid. The hypoxanthin presumably passes through an intermediate xanthin stage. According to their observations, these three enzymes are distributed in various tissue extracts in the following manner:—

Tissue.	Guanase.	Adenase.	Xantho-oxidase.
Liver of Ox . . .	+	+	+
„ Pig . . .	−	+	+
„ Rabbit . . .	+	−	+
„ Dog . . .	+	trace	−
Spleen of Dog . . .	+	+	+
„ Pig . . .	−	+	−
Pancreas of Pig . . .	+	+	−
„ Dog . .	−	+	−

According to the data of this table the distribution of the three enzymes is very irregular and capricious. The liver of one animal contains all three enzymes, that of another only two, and that of another only one. The same thing is true of the

[1] Schittenhelm, *Zeit. f. physiol. Chem.*, 42, p. 251, 1904; 43, p. 228, 1904.

[2] Jones and Austrian, *Zeit. f. physiol. Chem.*, 48, p. 110, 1906; Jones and Winternitz, *Zeit. f. physiol. Chem.*, 44, p. 1, 1905.

other organs, only we find, for instance, that dog's spleen contains all three enzymes, and pig's spleen only one of them, whilst dog's liver contains one, and pig's liver two. Schittenhelm and Schmidt[1] do not altogether accept this scheme of ferment distribution. Contrary to Jones and Austrian, they found that there was no lack of adenase in rabbit's liver. In fact, Schittenhelm[2] denies the necessity of assuming the independent existence of adenase and guanase enzymes, for he finds that any tissue extract which can desaminate the one amino purin can likewise attack the other. Still, he admits that the rate of action upon the two purins is very different in different cases, and that pig's spleen, for instance, acts upon adenin much more rapidly and completely than it does upon guanin, whilst dog's liver acts much more rapidly upon guanin than upon adenin. Jones, whilst admitting that some of the tissue extracts in which he found adenase or guanase to be lacking might contain traces of the enzyme, and so might exhibit some activity if only permitted to act for a long enough time, insists upon the existence of the two distinct enzymes. The evidence—admitted by Schittenhelm—as to the very different rates of action of different tissue extracts upon the two amino purins seems sufficient to uphold Jones's view, and, moreover, in some cases Jones failed to find any trace of enzyme, however long the tissue extract was permitted to act.

As regards the distribution of xantho-oxidase, Schittenhelm is in agreement with Jones. He found, for instance, that this enzyme is present in large amount in ox spleen, but is wanting in pig's spleen. Burian[3] made a preparation of the oxidase from minced ox liver which contained only traces of nucleoproteins and purin bodies, and on keeping it at 37° with xanthin and hypoxanthin in presence of oxygen, these substances were rapidly oxidised to uric acid, without the oxidase apparently undergoing any diminution of activity.

Accepting the experimental results given in the above table as substantially correct, we are by no means clear as to their interpretation. The simple and straightforward interpretation,

[1] Schittenhelm and Schmidt, *Zeit. f. physiol. Chem.*, 50, p. 30, 1906.
[2] Schittenhelm, *ibid.*, 45, p. 152; 46, p. 354, 1905.
[3] Burian, *ibid.*, 43, p. 497, 1904.

viz., that these results accurately indicate which enzymes were present in the tissues before their death and disintegration, and which were not, cannot be accepted with any confidence. It is, on the face of it, highly improbable that the distribution of the three enzymes in the various organs of one and the same animal should be so irregular, and still more so that the corresponding organs of another animal should show quite a different distribution. As will be pointed out more fully in a subsequent portion of this lecture, these intracellular enzymes must be of functional importance, and so they must be studied and re-studied in connection with their probable functions. They are presumably concerned in the conversion of the purin bodies derived from their own tissues—and in some cases those derived from external sources—into uric acid, in order that the uric acid, wholly or in part, may pass into the blood and be excreted from the system. Supposing, therefore, the nucleins of any given organ be found to contain either guanin or adenin, but not both of these purins, then one would naturally expect that an extract of that organ would, as a rule, contain only the corresponding enzyme. Conversely, if an organ extract be found to contain only adenase, or guanase, then one would expect that the nucleins of that organ would contain only adenin or guanin. Supposing that this relationship between organ enzyme and composition of organ nuclein is not established, as seems more than likely to be the case from the few data at present available, then one is driven to the conclusion that the simple and straightforward interpretation of experimental results above referred to is invalid. There are several possible ways in which apparent contradictions of this kind can arise. In the first place, the mechanical treatment of breaking up and extracting a tissue may be so violent as entirely to destroy some of the more unstable endoenzymes bound up in the tissues. Again, the action of some of the enzymes in an extract may be entirely neutralised by the presence of anti-bodies. Again, other enzymes may be present which do not interfere with the action of the enzyme under examination, but which convert the product formed by this latter enzyme, the amount of which is taken as a measure of its activity, into some other unknown substance.

As far as I am aware, a comparison between the nucleins

and the enzymes present in a tissue has been made in only one case. Schittenhelm[1] found that fresh pig's spleen contains adenin and guanin, and, as already mentioned, he likewise found that it contains both adenase and guanase. His result is almost certainly correct, therefore, but the fact that Jones failed to find any trace of guanase is a point requiring re-investigation. Until other comparisons have been made between the composition of the nucleic acids and the related enzymes of tissues, the question of their correspondence or otherwise must remain in abeyance.

Uricolytic Enzyme.—In addition to uric acid forming enzymes, most tissues contain also a uric acid destroying enzyme. The disintegrative action of animal tissues upon uric acid was noted by Stokvis as long ago as 1859. In recent years the subject has been studied by Chassevant and Richet,[2] Ascoli,[3] Jacoby,[4] Wiener,[5] Schittenhelm,[6] Burian,[7] Austin,[8] Almagia,[9] and by Wiechowski and Wiener.[10] Some of the results obtained by these observers are collected in tabular form, and it will be seen

Tissue.	Ox.	Horse.	Pig.	Dog.	Rabbit.
Liver . . .	+	+	+	+	+
Kidney . .	+	+	...	−	−
Spleen . .	+ −	+	...	...	...
Muscle . .	+	+	...	...	...
Bone marrow .	+ (?)	+	...	...	...
Intestine . .	−	...	...	...	...
Lung . . .	−	...	...	...	...

that most of the tissues examined were found to contain the enzyme. As in the case of the uric acid forming enzymes, however, the corresponding organs of different animals do not

[1] Schittenhelm, *Zeit. f. physiol. Chem.*, 46, p. 354, 1905.
[2] Chassevant and Richet, *C. R. Soc. Biol.*, 1897, p. 743.
[3] Ascoli, *Pflüger's Archiv.*, 72, p. 340, 1897.
[4] Jacoby, *Virchow's Arch.*, 157, p. 235, 1899.
[5] Wiener, *Arch. f. exp. Path.*, 42, p. 375, 1899.
[6] Schittenhelm, *Zeit. f. physiol. Chem.*, 45, pp. 121 and 161, 1905.
[7] Burian, *ibid.*, 43, pp. 487 and 532, 1904.
[8] Austin, *Journ. Med. Research*, 15, p. 309, and 16, p. 71.
[9] Almagia, *Hofmeister's Beitr.*, 7, p. 459, 1906.
[10] Wiechowski and Wiener, *ibid.*, 9, p. 247, 1907.

always agree. The kidney of the ox and horse contained the enzyme, but that of the dog and rabbit apparently did not. A result such as this must be accepted with reserve. In the case of ox spleen, Austin found the enzyme, whilst Schittenhelm was unable to detect it.

The uricolytic and uricogenic enzymes have nothing in common with one another, as they do not run parallel in various tissues. The uricolytic enzyme is a soluble body, which can be thrown out of solution by means of uranium acetate, and then isolated from the precipitate by digestion with ·2 per cent. sodium carbonate. Wiechowski and Wiener consider it to be an oxidase, as it acts best in presence of plenty of air. It is a moderately active body, for they found that 4 gms. of dried and powdered dog's liver, when kept with ·05 per cent. Na_2CO_3 solution and sodium urate at 40°, decomposed ·51 gm. of uric acid in four hours. Or again, the 100 gms. of dry powder obtained from the kidneys of an ox were able to split up about 70 gms. of uric acid in twenty-four hours.

The fate of the uric acid is not known with certainty. Chassevant and Richet thought that minced liver changed it into urea. Subkow,[1] after acting upon uric acid with dog's liver under anaërobic conditions, isolated urea. Wiener thought that under aerobic conditions some of it was converted into glycin. Jacoby found that dog's liver converted it into allantoin. Cipollina[2] concluded that the spleen, and perhaps also the liver and muscles, could oxidise it to oxalic acid. Almagia found that it was converted, first into allantoin, and then into glyoxylic acid. However, none of these observers offered a complete proof of the formation, from the uric acid, of the various products isolated by them. But Wiechowski[3] found that if sodium urate solution were shaken up for four to eight hours at 40° with purified emulsions of organs such as the liver and kidney, the whole of the urate was destroyed, and was in some cases oxidised quantitatively to allantoin. Hence there could have been no formation of urea or glycin, though possibly under anaërobic conditions such as Subkow used, the changes may have been

[1] Subkow, *Diss., Moscow*, 1903, cited by *Jahrb. f. Tierchem.*, 33, p. 873.
[2] Cipollina, *Berl. klin. Wochenschr.*, 1901, p. 544.
[3] Wiechowski, *Hofmeister's Beitr.*, 9, p. 295, 1907.

different. Or perhaps the allantoin formed first may have undergone a subsequent conversion into urea and other substances.

If purin bases be added to a suitable tissue extract, it follows that several changes will occur at one and the same time. The purins, if in the form of adenin and guanin, will gradually be converted into hypoxanthin and xanthin. These oxypurins, directly they are formed, will in turn be oxidised to uric acid. This uric acid will then undergo oxidation into allantoin, and this allantoin very probably will undergo further decomposition into urea, glycin, or glyoxylic acid. The course of these changes, so far as they concern the uric acid, has been examined in detail by Burian.[1] Under the particular conditions of experiment, in which a purified ox liver extract was allowed to digest a well oxygenated solution containing xanthin, the uric acid was found to increase to a maximum during the first three hours' digestion, and then to diminish gradually.

Intracellular Enzymes and Functional Capacity.—We have seen that so far as our meagre information avails us, the intracellular enzymes concerned in nucleoprotein metabolism do not correspond at all closely with the character of the work that one imagines they are called upon to perform. No quantitative determinations of the relative amounts of the enzymes in the various tissues have been made, though Almagia found that in uric acid destroying power the liver was the most active, and then in order came the kidney, lymph gland, muscle, bone marrow, spleen, and thyroid. The nucleoprotein content of these tissues has a very different order, or seems to bear no relationship to enzyme activity; but obviously the whole subject needs investigating in much greater detail before a definite opinion be adopted. In the case of certain other proteolytic enzymes we are a little better informed, though as yet only the fringe of the subject has been touched upon. The enzyme erepsin has been investigated by the writer[2] in some detail, as it is of universal distribution, and it seems to occur in fairly constant proportion in the tissues of very different classes of animals. From the mean results given in the table, we see that

[1] Burian, *Zeit. f. physiol. Chem.*, 43, p. 497, 1904.

[2] Vernon, *Journ. Physiol.*, 33, p. 81, 1905.

in the carnivorous hedgehog, the omnivorous cat and man, and in the herbivorous rabbit and guineapig, the extreme values of a tissue such as the liver vary only from 1·9 to 3·6. The kidney

Animal.	Hedge-hog.	Cat.	Man.	Rabbit.	Guinea-pig.
Number of Observations . .	2	8 to 10	2	8	7
Kidney	7·9	11·6	5·2	10·9	8·8
Liver	1·9	3·6	2·7	2·3	3·4
Cardiac muscle	·48	·95	·54	1·4	1·8
Skeletal muscle	·25	·56	(·18)	·46	·66
Brain	·23	1·0	(·27)	·49	·68

contains two to four times more erepsin than this, and the other tissues less than half as much; but having regard to the small number of observations made, and the great differences in the conditions of life of the animals examined, one is led to conclude that given similar conditions, the average ereptic value of a tissue may be roughly constant through a wide range of the mammalian kingdom. There can be little doubt, therefore, that this enzyme is closely bound up with certain proteolytic activities of the tissues. Perhaps its amount is directly proportional to the protein metabolism, but of this we know nothing at present.

Though the *average* amount of erepsin in a tissue is nearly constant, yet it varies very greatly with the functional activity of the tissue. This is strikingly indicated by the data in the table,

Age of Guineapig.	Fœtal.	0 day.	8 days.	31 days and over.
Weight . . .	37 gms.	57 gms.	81 gms.	585 gms.
Kidney . . .	1·8	6·8	8·0	8·9
Liver	1·6	2·6	3·3	3·3
Cardiac muscle . .	·48	·67	·70	1·6
Skeletal muscle . .	·56	·79	·90	·65
Brain	·45	·26	·64	·73

which show the effect of growth upon ereptic power. In the fœtal tissues the erepsin is at a minimum, and it rapidly increases as the animal develops, till it reaches its full value—in the

case of the guineapig—after about eight days of postnatal existence. Similar results were obtained with the rabbit and the cat, though in the case of the cat especially, the tissues did not reach their full ereptic power so soon after birth. This is probably connected with the fact that the kitten is much slower in attaining maturity than the young guineapig or rabbit. Very small fœtal rabbits, weighing only 4 gms. were found to have only about a tenth as much erepsin in their tissues as the fœtal guineapig above recorded, so presumably in a still earlier stage of development the embryo is almost enzyme-free. Battelli and Stern[1] obtained similar results to this in respect of the hydrogen peroxide decomposing ferment catalase. They found that the liver and kidney of the guineapig showed a rapid increase of enzyme during embryonic development, and the first few days after birth, but that subsequent to the seventh day there was little or no further increase. At this period the liver contained five times more ferment than that of the new-born guineapig, whilst the kidney contained twice as much. The other tissues examined, viz., the spleen, lung, muscle, and brain, showed only a slight increase of enzyme with growth.

Analogous results have been obtained by Jones and Austrian[2] in respect of the nuclein ferments. They found that in the earlier stages of fœtal development, the liver of pigs contained no nuclein ferments whatever. Embryos less than 150 mm. in length had no appreciable quantity of adenase, but those of 165 to 200 mm. had a distinct amount. Xantho-oxidase did not appear till a later period, perhaps after birth, whilst guanase was invariably wanting, both before and after birth. Mendel and Mitchell[3] made independent observations with pigs' embryos averaging 50, 75, and 100 mm. in length. They found adenase in the liver of even the smallest embryos, but as far as one can judge from their incomplete data, it distinctly increased in amount with growth. As in Jones and Austrian's experiments, no xantho-oxidase or guanase were found in the liver, but an extract of the other viscera contained guanase.

[1] Battelli and Stern, *Archiv. d. Fisiologia*, 2, p. 471, 1905.
[2] Jones and Austrian, *Journ. Biol. Chem.*, 3, p. 227, 1907.
[3] Mendel and Mitchell, *Amer. Journ. Physiol.*, 21, p. 69, 1908.

Other observations, upon the fat- and carbohydrate-splitting enzymes of embryos, are recorded in the next lecture. These enzymes increase with the growth of the embryo in the same way as the proteolytic enzymes.

In apparent contradiction to these conclusions is the work of Schlesinger,[1] who found that in new-born rabbits the autolysis of ground-up liver tissue was maximal, whilst in eight-day rabbits it was considerably greater than in older animals. Again, Mendel and Leavenworth[2] investigated the autolysis of pig's liver, and they found that in the presence of ·02 per cent. acetic acid the minced liver of embryos 50 to 280 mm. in length showed slightly more autolysis than that of adult pigs; but when no acid was added, the embryo liver hydrolysed only a third or fourth as much of its proteins to the non-coagulable stage as the adult liver. In considering these results, one must remember that the tissues of young animals are much more fragile and easily digestible than those of older ones, and so would more readily undergo autolysis in spite of a deficiency of enzyme. The only valid method of comparing the enzyme activity of tissues from animals of various ages is to separate the enzymes as far as possible from the tissue proteins (*e.g.*, by making glycerin extracts) and test their action on fibrin, caseinogen, or some other foreign protein.

The explanation of all these results seems clear. Embryonic animals receive most of their food ready formed, in an easily assimilable condition, and do not need to elaborate it for themselves. Even after birth the new-born animals are lacking in vigour for some days, and they are feeding on the easily digestible maternal milk; so their tissues, though more active than in the embryonic state, are not functioning so completely as later on, when they have to perform the same duties as those of the full-grown animals. In growing animals, therefore, the ereptic power of the tissues is closely related to their functional activity and functional capacity.

The ereptic power of the tissues is moderately affected by diet. I found that cats fed for eleven to twenty-nine days on a meat and fat diet had on an average 1·3 times more erepsin

[1] Schlesinger, *Hofmeister's Beitr.*, 4, p. 87, 1904.

[2] Mendel and Leavenworth, *Amer. Journ. Physiol.*, 21, p. 69, 1908.

in their tissues than cats fed on bread and milk diet, whilst cats kept on a mixed diet had most enzyme of all. The response of the intracellular enzymes to changes of nutrition, just as that of the extracellular ones,[1] is only a slow and gradual one, and is not marked as a rule. Probably in cases of starvation they would be more striking, but I made no direct observations to test the point. However, I compared the tissues of hibernating hedgehogs with those of non-hibernating, and a comparison of the mean results given in the table shows that most of the

Tissue.	Non-hibernating Hedgehogs.	Hibernating Hedgehogs.	Ratio.
Kidney	7·9	3·8	2·1 : 1
Pancreas	3·0	3·0	1·0
Spleen	5·2	·70	7·4
Liver	1·9	·76	2·5
Cardiac muscle . .	·48	·30	1·6
Skeletal muscle . .	·25	·24	1·0
Brain	·23	·23	1·0

tissues of the active animals were considerably richer in erepsin than those of the passive ones. The spleen is especially noticeable, as it contained seven times more ferment in the one case than in the other. The differences of ereptic power are not immediately connected with the differences of temperature of the hibernating and the non-hibernating animals, for one of the hibernating hedgehogs was examined and found to have a temperature of 9·2°, but it was not killed till the following day. By that time its temperature had shot up to 35°, yet this animal had practically the same amounts of erepsin in its tissues as another animal which was killed when its temperature was 7·5°. Probably, therefore, the diminution in the ereptic power of the tissues only gradually ensues in the hibernating hedgehogs after the onset of the winter sleep, whilst the increase which must occur when the animal becomes active again in the spring is likewise a gradual one.

The effect of disease on ereptic power may be very great. As we see from the data in the table, guineapigs which had wasted away gradually for some weeks before death, and were

[1] *Cf.* Vassiliew, *Arch. d. Sci. Biol.*, 2, p. 219, 1893.

reduced to less than half the normal weight, contained less than half as much erepsin in their tissues as healthy animals, though a guineapig which died three days after a *Staphylococcus* injection, and which consequently had had no time to waste away, showed no defect of the enzyme. Again, in the case of man,

Tissue.	Normal Guineapigs (767 grms.).	Wasted Guineapigs (305 grms.).	Died 3 days after *Staphylococcus* injection (577 grms.).
Kidney . . .	8·9	3·7	7·8
Liver . . .	3·3	1·4	3·6
Cardiac muscle .	1·9	·72	2·8
Skeletal muscle .	·65	·59	·72
Brain . . .	·73	·31	·41

I found that healthy kidneys had an average ereptic value of 5·4. Slightly diseased kidneys showing some cloudy swelling or fatty degeneration, had a value of 3·4. Kidneys showing interstitial nephritis had one of 2·8, and a kidney with very advanced nephritis one of only ·86. Here again ereptic power was very closely correlated with functional activity and capacity.

What is true of erepsin is probably true of the other proteolytic endoenzymes of the tissues, though there is but little exact information upon the subject. In so far as one can deduce comparable data, the relative amounts of β-protease enzyme found by Hedin and Rowland[1] in the press juice from the tissues of the ox, correspond moderately well with the relative amounts of erepsin in the tissues of the cat. The figures given

Tissue.	Ereptic Values of Cat's Tissues.	Per cent. Protein hydrolysed in Acid Solution (β-protease).
Spleen	7·6	89
Pancreas	6·4	about 90
Liver	5·0	50
Cardiac muscle . . .	1·6	about 45
Skeletal muscle . . .	·8	slight
Submaxillary gland . .	5·3	very slight
Blood	·3	very slight
Serum	·1	nil

[1] Hedin and Rowland, *Zeit. f. physiol. Chem.*, 32, pp. 341 and 531, 1901.

in the table for β-protease represent the percentage of protein hydrolysed to or beyond the peptone stage when digesting in the presence of ·25 per cent. of acetic acid. The only real lack of correspondence is shown by the submaxillary gland, and of course a gland such as this might vary considerably in different animals.

The variation of proteolytic endoenzymes with functional activity is shown by the observations of Hildebrandt,[1] who concluded that the proteolytic ferments in the cow's mammary gland at the height of its activity and secretory powers are greater in amount than in non-secreting or feebly secreting glands. Also the mammary glands of seven women who had died after childbirth were found by him to contain much more active proteolytic enzymes than those of non-pregnant women. The nitrogen hydrolysed to or beyond the albumose stage by autolysis was about four times greater in the former case than in the latter. Again, in correspondence with the diminished functional activity produced by wasting disease, Schlesinger[2] found that the autolytic power of minced liver tissue varied with the degree of atrophy at the time of death. In children who weighed only 35 per cent. of their normal amount, the autolysis was only half as great as in those who weighed 80 per cent. of the normal. Brugsch and Schittenhelm[3] conclude that in gouty patients the whole series of ferments connected with purin metabolism is in a condition of enfeebled activity. They found that such patients, when fed with nucleic acid, were much slower than normal people in metabolising it and excreting the uric acid formed.

Special Endoenzymes.—In addition to the numerous proteolytic endoenzymes which are common to all tissues, and which are concerned in the hydrolysis of proteins and their normal decomposition products along well-known lines, it is probable that certain organs and tissues possess other enzymes more or less peculiar to themselves, or at any rate not of universal distribution. These enzymes act upon nitrogenous bodies which are formed by what may be termed the abnormal

[1] Hildebrandt, *Hofmeister's Beitr.*, 5, p. 463, 1904.
[2] Schlesinger, *loc. cit.*
[3] Brugsch and Schittenhelm, *Zeit. f. exp. Path. u. Therap.*, 4, p. 438.

cleavage of the protein molecule, or in other ways. Two of the most interesting of such nitrogenous bodies are creatin and hippuric acid. As far as is known, creatin is found in appreciable quantity only in muscle, and to a less extent, in nervous tissue. Hence, if the hypothesis of correlation between endoenzyme and functional capacity be valid, one would expect to find creatin-forming and creatin-destroying enzymes in these two tissues, but not in the other tissues of the body. The evidence is very incomplete, but so far as it goes, it must be admitted that it does not give much support to the hypothesis. Gottlieb and Stangassinger[1] found that many tissues such as muscle, liver, kidney, spleen, and suprarenal gland contain an enzyme which gradually converts creatin into creatinin, and other enzymes which destroy both creatin and creatinin. The press juice of muscle, and to a less extent that of the kidney, was found to contain in addition a creatin-forming enzyme. Hence it follows that the amounts of creatin and creatinin present in press juice undergoing autolysis gradually rise or fall, according to the relative rates at which the four enzymes are exerting their activities. For instance, in one experiment the press juice of muscle (to which some creatin had been added) showed an increase of its total creatin *plus* creatinin from ·39 per cent. to a maximum ·63 per cent. after forty hours' autolysis, and then a gradual diminution down to ·33 per cent. after ninety-one days. No evidence of the existence of a creatin-forming enzyme in extracts of the tissues was obtained, and observations upon press juice do not seem to have extended beyond the two tissues mentioned. Further investigation upon the press juice of other tissues might show that they contained no creatin-forming enzyme, or very much less than is present in muscle, and hence might conform to the hypothesis of correlation between endoenzyme and functional activity. The widely distributed creatin- and creatinin-destroying enzymes are probably not specific bodies of limited activity, but may be identical with the enzyme or enzymes described in the last lecture, which hydrolyse glycin, leucin, tyrosin, and other amino acids. They are distinct from arginase, for Dakin[2] states that this enzyme is without

[1] Gottlieb and Stangassinger, *Zeit. f. physiol. Chem.*, 52, p. 1, 1907.

[2] Dakin, *Journ. Biol. Chem.*, 3, p. 435, 1907.

action upon creatin and creatinin. But at present it is best to suspend judgment upon the whole question of these enzymes, for Mellanby[1] has recently repeated some of Gottlieb and Stangassinger's experiments, and he has been unable to confirm them in any single particular. He found that if creatin and creatinin were kept with extracts of liver or muscle for two or three days at 37° in presence of toluol, they underwent no change whatever, whilst the creatin content of muscle which was allowed to undergo autolysis aseptically or antiseptically, was likewise unchanged. The conversion of creatin to creatinin observed by Gottlieb and Stangassinger in some of their experiments is attributed by Mellanby to the fact that they evaporated to dryness on the water-bath, a process known to lead to a conversion of creatin to creatinin.

The reaction of hippuric acid to endoenzymes has been known since 1881, when Schmiedeberg[2] described a "histozym" which could decompose it. This ferment was found by him in moderate quantity in pig's blood, and in the liver of the dog and kidney of the pig, whilst the kidney of the dog and lung of the pig contained a small amount of it. Nencki and Blank[3] found that pancreatic extracts decomposed hippuric acid, but Gulewitsch[4] and Schwarzschild[5] showed that pepsin and trypsin do not attack it, whilst Cohnheim[6] found that it likewise resisted the action of erepsin.

The hippuric acid-splitting power of liver juice has been demonstrated by Jacoby,[7] and that of minced kidney by Berninzone[8] and by Wingler.[9] No adequate comparative observations upon the hippuric acid-splitting power of the tissues appear to have been made, but probably it would be found that organs other than the liver and kidney, if they contain the enzyme at all, have it in but small quantity.

[1] Mellanby, *Journ. Physiol.*, 36, p. 447, 1908.
[2] Schmiedeberg, *Arch. f. exp. Path.*, 14, p. 379, 1881.
[3] Nencki and Blank, *ibid.*, 20, p. 367, 1886.
[4] Gulewitsch, *Zeit. f. physiol. Chem.*, 27, p. 540, 1899.
[5] Schwarzschild, *Hofmeister's Beitr.*, 4, p. 155, 1904.
[6] Cohnheim, *Zeit. f. physiol. Chem.*, 52, p. 526, 1907.
[7] Jacoby, *ibid.*, 30, p. 149, 1900.
[8] Berninzone, *Atti. d. Soc. ligust. d. Sci. nat.*, xi., 1900.
[9] Wingler, *Inaug. Diss. Abstr.*, in Maly's *Jahresb.*, p. 92, 1900.

Proteolytic Endoenzymes in Lower Animals.—Most of the evidence concerning endoenzymes adduced thus far concerns mammalian tissues, but what is true of one living tissue is probably true to a greater or less extent of all, and so far as has been investigated the tissues of every living organism, vegetable no less than animal, contain proteolytic endoenzymes.

In the protozoa it is self-evident that all digestion must be of an intracellular nature, but it is often comparable to the extracellular digestion of higher animals, in that a watery vacuole is formed around the particle of ingested food, and enzymes are secreted by the protoplasm of the organism into this vacuole. It was noted by Engelmann [1] that granules of blue litmus, if ingested by Amœbæ and Paramoecia, were changed to a red colour in a few minutes. Brandt [2] tested the intracellular staining reactions of amœbæ by means of hæmatoxylin, and likewise found that the watery vacuoles contained acid. Metschnikoff [3] observed that the plasmodia of different species of Mycetozoa secreted an acid liquid round ingested litmus grains. Miss Greenwood and Miss Saunders [4] made numerous observations upon the Infusorian, *Carchesium Polypinum*, and the plasmodia of certain Mycetozoa, and they found that the ingestion of solid matter, whatever its nature, stimulates the surrounding cell substance to secrete acid fluid. A definite watery vacuole need not necessarily be formed, for in the case of the Mycetozoa the ingested mass is as a rule merely permeated with acid. The vacuole, after a time, gradually loses its free acid, and finally the ingested litmus shows a neutral or alkaline reaction.

These results are of importance in their bearing upon the endoenzymes of higher animals. We saw in the previous lecture that the most powerful proteolytic enzyme of the tissues acts in an acid medium, not an alkaline one. Hence, it is possible that intracellular digestion occurs in higher animals, just as in the Protozoa, by the secretion of an acid liquid and of the appropriate acid-acting enzyme around the particle of protein matter which the cell desires to break down. It seems

[1] Engelmann, *Hermann's Handbuch d. Physiol.*, 1, p. 349, 1878.
[2] Brandt, *Biol. Centralb.*, 1, p. 202, 1881.
[3] Metschnikoff, *Ann. de l'Inst. Pasteur*, 1889, p. 25.
[4] Greenwood and Saunders, *Journ. Physiol.*, 16, p. 441, 1884.

improbable, however, that this is the normal mechanism of degradation of protein matter which is always going on in all tissues, and which forms a large part of the protein metabolism of a normal animal, and the whole of it in a starving one. It is much more probable that the intracellular enzymes exert their activities when still attached to and forming part of the structure of the living protoplasm of the cell. A local acid environment might still be induced, however, whereby they could act upon neighbouring particles of protein matter under the most favourable conditions; but such surmises are useless in the present state of knowledge.

The fact that the watery vacuoles were noticed to assume ultimately a neutral or alkaline reaction, is likewise a significant one, for it implies that the endoerepsin, which is chiefly an alkali-acting enzyme, would find itself under the most suitable conditions for carrying on and completing the digestion of the food substance which had been broken down in its earlier stages by the acid-acting β-protease.

It is stated by Krukenberg[1] that glycerin extracts of the Mycetozoa plasmodia contain a very active "peptic" enzyme, *i.e.*, an enzyme which digests in an acid medium, but not in an alkaline one. It is sufficiently powerful to digest boiled fibrin. Krukenberg did not determine whether it could split up peptones to the amino acid stage, but probably it would be found to resemble β-protease rather than pepsin.

In the sponges and many of the Cœlentera[2] digestion is mainly intracellular, as in the Protozoa. Krukenberg[3] found that glycerin extracts of the parenchyma of many different species of sponges could digest raw fibrin in an alkaline medium, as well as in an acid one. Frédéricq[4] found a tryptic ferment in Actinia, whilst Krukenberg[5] found that they contained an intra-

[1] Krukenberg, *Untersuch. d. Physiol. Inst. Heidelberg*, 2, p. 273, 1878.

[2] *Cf.* von Fürth, *Vergleichende Chemische Physiologie der niederen Tiere*, Jena, 1903, pp. 153 and 161. The whole subject of digestion in invertebrate animals, both intracellular and extracellular, is dealt with fully in this book.

[3] Krukenberg, *Vergl. Studien*, 1 Reibe, 1 Abt., 1880, p. 64 ; *Untersuch. d. Physiol. Inst. Heidelberg*, 2, p. 339, 1882.

[4] Frédéricq, *Bull. Acad. Roy. de Belgique*, 47, 1878.

[5] Krukenberg, *Untersuch. d. Physiol. Inst. Heidelberg*, 2, p. 338, 1882.

cellular acid-acting ferment. Mesnil[1] found that an aqueous extract of ground-up filaments of the mesenteries of Actinia contained a proteolytic enzyme which acted both in acid and in alkaline solution. It acted best at 36°, and both tyrosin and tryptophan could be detected among the products of digestion.

Like Protozoa and Mycetozoa, the cells of sponges and Actinia can secrete acid intracellularly. Loisel[2] observed that litmus grains ingested by the parenchyma cells of living sponges showed an acid reaction, whilst Chapeaux[3] observed an intracellular secretion of acid in Actinia.

Most of the other observations upon the enzymes of invertebrate animals were made with extracts of the alimentary canal, and of the glands pouring secretions into it, and so concern exoenzymes rather than endoenzymes. Still, judging from the observations above described, there can be no doubt that the tissues of the lower animals contain both acid- and alkali-acting proteases of a similar nature to those found in higher animals. They likewise contain enzymes of an erepsin-like nature, *i.e.*, enzymes which hydrolyse proteoses and peptones, but which have little or no action upon native proteins. Thus I found[4] that the tissues of the frog, for instance, contained as a rule about a third as much erepsin as the corresponding tissues of mammals, whilst those of the eel contained a fifth to a tenth as much. The tissues of the lobster were about equally poor in ferment, whilst those of the fresh water mussel *Anodon* contained much less still. To test the character of the erepsin, comparative digests were carried out in acid as well as in alkaline media. In the presence of ·1 per cent. of acetic acid, I found that extracts of cats' tissues, on an average, digested Witte's peptone forty-two times more slowly than in presence of ·1 per cent. Na_2CO_3—*i.e.*, the erepsin is almost entirely an alkali-acting one. Extracts of the tissues of the frog, eel, lobster, and Anodon, on the other hand, digested on an average only five times more slowly in acid solution than in alkaline, and with

[1] Mesnil, *Ann. de. l'Inst. Pasteur*, 15, p. 352, 1901.
[2] Loisel, *Journ. Anat. Physiol.*, 34, p. 187, 1897.
[3] Chapeaux, *Arch. de Zool. Exp.* (3), 1, p. 139, 1893.
[4] Vernon, *Journ. Physiol.*, 32, p. 33, 1904.

three extracts the digestion rate was only 1·4 to 2·0 times slower. Evidently, therefore, the erepsin is relatively much less affected by acidity and alkalinity than that of mammalian tissues.

Proteolytic Endoenzymes in Plants.—In plant tissues, so far as they have been investigated, the proteolytic enzymes act better in an acid medium than in an alkaline one. Vines[1] made a very extensive series of observations upon the proteolytic enzymes of plants of many different Natural Orders, and he finds that a peptolysing or erepsin-like enzyme is present in every plant, and as a rule in every part of the plant; leaves, stems, roots, bulbs, tubers, fruits, and seeds. The enzyme digests Witte's peptone best at the natural acidity of the plant juice, but it can also act, though less vigorously, through a fairly wide range of acid and alkaline reaction. For instance, an aqueous extract of pulped mushroom digested best at its natural acidity, and when neutralised with calcium carbonate: less well in presence of 1·25 per cent. Na_2CO_3 or in ·1 per cent. HCl, and feebly in ·2 per cent. HCl. Again, an aqueous extract of pounded barley which had been allowed to germinate for eleven days, digested best at its natural acidity, less well in presence of ·5 per cent. Na_2CO_3, and feebly in presence of ·2 per cent. HCl. On the other hand, a glycerin extract of Dahlia root digested best in presence of ·5 per cent. citric acid, less well at natural acidity, and feebly in presence of ·5 per cent. Na_2CO_3.

We see, therefore, that the endoerepsins of vegetable tissues, though very different from those of the lower animals examined, differ from them less widely than from those of the higher animals. Hence it seems possible that the enzymes of the lowest members of the animal kingdom will be found to differ still less, and may show comparatively little preference for an alkaline medium as against an acid one.

Though endoerepsins are probably of universal occurrence in plants, it seems probable that enzymes capable of digesting the higher proteins are of more restricted distribution, or at any rate they are frequently present in such small amount that it is not possible to test for them. Such enzymes, sometimes known

[1] Vines, *Annals of Botany*, 15, p. 563, 1901; 16, p. 1, 1902; 17, p. 237, 1903; 18, p. 289, 1904; 19, p. 171, 1905; 20, p. 113, 1906.

as vegetable trypsins,[1] have been recognised and worked upon for years. They were first described by Gorup-Besanez[2] in germinating seeds in 1874, though he regarded them as rather of a peptic than a tryptic nature. The enzymes which have been examined in most detail are bromelin, from the fruit of the pine-apple: papain, from the fruit of the papaw (*Carica papaya*): cradein, from the latex of the fig, and the endotrypsin of yeast. This last is a somewhat active body, but as a rule the vegetable enzymes are very weak compared with those of animal origin. The term "vegetable trypsin" was adopted because these plant enzymes, like animal trypsin and in contradistinction to pepsin, split up proteins into leucin, tyrosin, tryptophan, and other decomposition products. But this was before the existence of more than one class of proteolytic enzymes was recognised, and in the light of modern knowledge Vines[3] regards the protein-digesting or peptonising enzyme of plants as more comparable to pepsin than trypsin, and attributes their peptone-splitting power to an entirely distinct vegetable erepsin. He has succeeded[4] in separating the peptase and ereptase enzymes of hemp seed (*Cannabis sativa*). He extracted the crushed seeds with 10 per cent. NaCl solution, and faintly acidified the extract with acetic acid. The precipitate thrown down was washed with acid saline, and when dissolved in water gave a solution which digested fibrin, but had no action upon Witte's peptone. On the other hand the filtrate from the precipitate contained ereptase, but no peptase.

Professor Vines informs me that he has recently effected a separation of the peptonising and peptolytic enzymes of papain. He first extracted papain powder with twenty-five times its weight of 2 per cent. NaCl, whereby most of the protein and erepsin, and much of the peptonising ferment, were removed. The residue was then washed with 20 parts of water, whereby the remainder of the protein and erepsin still present was got rid of. A further extraction of the residue with 2 per cent.

[1] *Cf.* Reynolds Green, *The Soluble Ferments and Fermentation*, Cambridge, 2nd ed., chap. xiii., 1901.

[2] Gorup-Besanez, *Sitzber. d. phys. med. Soc. zu. Erlangen*, 1874, p. 75.

[3] Vines, *loc. cit.*, 20, p. 121, 1906.

[4] Vines, *Ann. Bot.*, 22, p. 103, 1908.

NaCl now gave a solution which could digest fibrin in twenty-four hours, but which was unable to split up Witte's peptone into tryptophan and other decomposition products. That is to say, it contained a peptonising ferment, but not a peptolytic one. The peptolytic enzyme was most active in acid media, whilst the peptonising enzyme digested well in a neutral or slightly alkaline medium, or in the presence of ·5 per cent. of an organic acid such as citric acid, but was inhibited in its action by ·05 to ·1 per cent. HCl.

The function of proteolytic endoenzymes in plants is similar to that in animals. They are concerned in the protein metabolism of the tissues, and in rendering the supplies of insoluble and indiffusible nitrogenous food material which may be stored up in certain parts of the plant, available for the whole organism whenever they are needed. For this purpose they must be dissolved, and to effect this solution proteolytic endoenzymes are requisite. To quote Professor Vines,[1] "their importance is strikingly illustrated in a germinating seed, where the reserve materials, whether deposited in the cotyledons or in the endosperm, have to be made available for the nutrition of the growing embryo." We accordingly find that as the reserve stores of protein are called upon in increasing degree, so the endoenzymes which render them effective are correspondingly elaborated. Reynolds Green[2] observed that the resting seed of the lupin (*Lupinus hirsutus*) did not yield any active enzyme to glycerin, but the ground cotyledons of seeds which had been allowed to germinate for four days, yielded an extract containing a fibrin-digesting enzyme. This enzyme worked best in presence of ·2 per cent. HCl, an acidity which corresponds roughly to the natural acidity of the germinating seeds. The enzyme was inactive in presence of weak alkalis, and ·5 per cent. Na_2CO_3 destroyed it entirely. Neumeister[3] made observations upon seedlings of the poppy, barley, wheat, maize, and rape. No enzyme was present in the early stages of germination, but it developed with growth of the plants, and reached a maximum when they had attained a length of 15 to 20 cm. The enzyme

[1] Vines, *Proc. Linn. Soc.*, 1903, p. 16.
[2] Green, *Phil. Trans. Roy. Soc.*, 178 B., p. 39, 1887.
[3] Neumeister, *Zeit. Biol.*, 30, p. 447, 1894.

digested only in acid liquids. An organic acid was necessary, oxalic acid being the best. Mineral acids such as HCl destroyed it. Vines[1] found that ungerminated seeds of the pea, broad bean, scarlet runner, white haricot bean, blue lupin, and maize, contain an ereptase, and also an enzyme which acts slowly on the reserve proteins of the seeds, but scarcely at all upon fibrin. The germinated seeds, on the other hand, contained in addition a weak fibrin-digesting enzyme. And lastly Bruschi[2] made observations upon the seeds of the castor oil plant, and found that the endosperm of ungerminated seeds was unable to undergo autolysis: but directly germination began, autolytic power developed.

[1] Vines, *Ann. Bot.*, 20, p. 113, 1906.

[2] Bruschi, *Rendic. d. R. Accad. d. Lincei* (5*a*), xv., 9, p. 563.

LECTURE III

FAT- AND CARBOHYDRATE-SPLITTING ENDOENZYMES

Lipolytic endoenzymes in animal tissues. Action upon esters, and upon natural fats. Vegetable lipolytic enzymes, and their relation to acids and alkalis. Glycogen content of tissues of adult and embryonic animals in relation to intracellular amylase. Maltase, invertase, and lactase in animal tissues. Lactase and adaptation. Vegetable diastatic and sucroclastic enzymes. Glucoside-splitting enzymes.

Lipolytic Endoenzymes.—Our knowledge of intracellular fat-splitting enzymes in animal tissues is in a somewhat fragmentary state. It is only within the last year or two that direct proof has been afforded of the existence of enzymes capable of hydrolysing the glycerides of the higher fatty acids. Previous to this, all observations had been made, not upon naturally occurring fats, but upon artificially prepared esters. That this is by no means the same thing has been emphasised by Connstein,[1] who showed that the lipase of the castor-oil plant acts best upon natural fats, and hardly attacks other ethereal salts at all.

The existence of lipolytic enzymes in animal tissues other than the pancreas was first demonstrated by Nencki and Lüdy[2] in 1887. The action of various tissue extracts upon the ester salol (phenyl salicylate) was tested, both in faintly acid and faintly alkaline solution. As can be seen from the data in the table, about two to four times more salol was split up in an alkaline medium than in an acid one. Arguing from these

[1] Connstein, "Ergebnisse der Physiol.," *Biochemie*, 3, p. 194, 1904.

[2] Nencki and Lüdy, *Therapeut. Monatshefte*, 1887, p. 417, quoted from Connstein, *ibid.*

Tissue.	Acid Solution.	Alkaline Solution.
	Per cent.	Per cent.
Pancreas	16·3	24·9
Liver	6·4	24·8
Intestinal mucosa . .	6·0	25·0
Stomach	5·1	11·1
Muscle	5·6	24·2

results, Nencki concluded that all tissues possess fat-splitting properties.

The next observations were made by Hanriot,[1] who allowed extracts of the minced tissues, previously neutralised with sodium carbonate, to act upon monobutyrin. The formation of butyric acid and glycerin was proved by the acid reaction which developed. The lipolytic enzyme was present in considerable quantity in the liver, and in small amount in the spleen and suprarenal gland, whilst from muscle, testis, and thyroid gland it was absent. The blood serum was very rich in it, for one drop of eel's serum (which contained five to seventeen times more enzyme than the serum of other animals) split up ·17 gm. of monobutyrin. The enzyme could split up the ethyl esters of formic, acetic, propionic, and isobutyric acids, and Hanriot also found that in three days it could completely hydrolyse the neutral fat present in the blood. Arthus[2] confirmed Hanriot with regard to the action of blood upon monobutyrin, but denied that it has any action upon olein, palmitin, and stearin.

The action of various tissue extracts on ethyl butyrate was studied in detail by Kastle and Loevenhart.[3] These observers ground up the tissue with sand, extracted it with five or ten times its volume of water, and allowed the extract to act upon ethyl butyrate. The free acid was estimated by titration with *N*/20 KOH. When mixtures of 4 c.c. of water, 1 c.c. of extract, ·26 c.c. of ethyl butyrate, and ·1 c.c. of toluol were kept for forty

[1] Hanriot, *C. R. Soc. Biol.*, 48, p. 925, 1896; *Comptes Rendus*, 123, p. 753, and 124, p. 778, 1897; *Arch. de Physiol.* (5), 10, p. 797, 1898.

[2] Arthus, *Journ. de Physiol.*, 4, pp. 56 and 455, 1902.

[3] Kastle and Loevenhart, *Amer. Chem. Journ.*, 24, p. 491, 1900.

minutes at 40°, the following amounts of butyric acid were liberated:

	Per cent.		Per cent.
Liver of pig . . .	9·5	Liver of chicken . .	1·95
„ sheep . . .	4·8	Pancreas of pig . . .	3·5
„ duck . . .	2·7	Kidney of pig . . .	1·8
„ ox . . .	2·2	Submaxillary gland of pig .	1·3

We see that liver extracts were the most active. That of the pig hydrolysed three times more ester than the corresponding pancreatic extract. Extract of the mucosa scraped from the duodenum of the pig's intestine hydrolysed 4·1 per cent. of the butyrate in thirty minutes, whilst extract of gastric mucous membrane had a smaller action. Comparative experiments made with the ethereal salts of the four lowest members of the fatty acid series showed that in fifteen minutes a pancreatic extract split up 1·75 per cent. of ethyl formate: 1·75 per cent. of ethyl acetate: 2·87 per cent. of ethyl propionate, and 4·37 per cent. of ethyl butyrate. It might be supposed, therefore, that the higher fatty acid compounds would be split up even more readily, but Kastle and Loevenhart found that they were acted on very much more slowly than ethyl butyrate.

The lipolytic enzyme appears to cling to a large extent to the solid particles of tissue cells. For instance, a liver extract which has been strained through cloth, and so contained such particles, split up 6·3 per cent. of the ethyl butyrate, but after it had been repeatedly filtered through filter paper, it split up only 2·8 per cent.

The lipolytic action of liver press juice (pig) upon the methyl, ethyl, amyl, and benzyl esters of optically inactive mandelic acid, $C_6H_5.CHOH.COOH$, has been studied by Dakin.[1] The action was rather feeble, but in every case Dakin found that the rate of hydrolysis of the dextro-rotatory component was greater than that of the lævo-rotatory component, so that if the hydrolysis were incomplete, an excess of free dextro-rotatory acid was liberated, and a residue of lævo-rotatory ester left.

The first adequate evidence of the existence of endocellular

[1] Dakin, *Journ. Physiol.*, 30, p. 253, 1904; 32, p. 199, 1905.

lipases capable of hydrolysing natural fats was obtained by Umber and Brugsch.[1] In their experiments, the whole of the body of the animal was washed out by injecting saline into the jugular vein. The various organs were rubbed up with kieselguhr, and the juice pressed out. This juice was allowed to act upon an emulsion of yolk of egg, in presence of ·25 to ·5 per cent. Na_2CO_3. Volumes of 2 c.c. of the organ juice were put with 5 c.c. of emulsion for fifteen to twenty-two hours at 37°, and, as can be seen from the data in the table, from 18·6 to 43·9 per cent.

	Fasting Dog.	Digesting Dog.
	Per cent.	Per cent.
Pancreas juice	26·7	43·9
Liver juice	37·0	19·5
Spleen juice	...	40·4
Intestinal mucosa juice	37·4	19·0
Serum	18·6	20·2
Corpuscles		19·0

of the fat was thereby split up. In the case of the fasting dog, the liver and intestinal mucosa juices were considerably more active than the pancreas juice, but in the dog killed whilst digesting a meat and fat meal, the pancreas juice was the most active. In that the fat digestion of the intestine is chiefly dependent upon the pancreas, it seems remarkable that its juice should not always possess much greater lipolytic activity than that of any other organ. It must be borne in mind, however, that we know nothing about the condition of pancreatic steapsin before secretion. It may exist in zymogen form, and for the most part continue so to exist in the expressed juice of the gland, just as the trypsin does unless specially activated. The considerably greater lipolytic power of the pancreas juice of the actively digesting dog may have been due to the presence of a certain amount of secreted steapsin, in addition to the normal intracellular lipase.

Umber and Brugsch also made observations upon the lipolytic powers of mixtures of their tissue juices. The most striking results were obtained by acting upon 5 c.c. of emulsion

[1] Umber and Brugsch, *Arch. f. exp. Path.*, 55, p. 164, 1906.

with 2 c.c. of pancreas juice *plus* 2 c.c. of liver juice of the fasting dog, when 71·8 per cent. of the fat was hydrolysed; whilst with 2 c.c. of pancreas juice *plus* 2 c.c. of spleen juice of the digesting dog no less than 82·1 per cent. of the fat was hydrolysed. This seems to show the existence of activating bodies in one or other of the organ juices, but unfortunately none of the experiments described were repeated, and so it is not permissible for us to draw far-reaching conclusions from them.

It is probable that the lipolytic power of the tissues increases during embryonic development in the same way as their proteolytic power. Wohlgemuth[1] found that if egg yolk were shaken with water and toluol, and allowed to undergo autolysis for four to ten weeks at 38°, it contained free glycerin, phosphoric acid, and cholin. This was presumably formed by the action of a lipolytic enzyme on the lecithin of the yolk: but a positive result was obtained only in five out of eight experiments, hence the amount of enzyme present is probably very small in all cases. Buxton and Shaffer[2] found a trace of lipase in very small embryos of the pig, rabbit, and sheep, and they state that the amount of enzyme increases with the age of the embryos. Mendel and Leavenworth[3] found that aqueous extracts of the ground-up liver and intestine of pig's embryos in every case had some hydrolytic action upon ethyl butyrate, but they were not nearly so active as the extracts of the corresponding tissues of adult pigs. For instance, extracts of the liver of embyros 50 to 75 mm. in length hydrolysed 5·4 per cent. of the ester on an average: those of the liver of embryos 150 to 215 mm. in length, 7·8 per cent., and those of the liver of adult pigs, 28·6 per cent.

Upon the lower animals scarcely any observations of lipolytic enzymes have been made. Cotte[4] describes a fat-splitting ferment in sponges, and Mesnil[5] found one in aqueous extracts of the ground-up mesentery filaments of Actinia, but no other

[1] Wohlgemuth, *Zeit. f. physiol. Chem.*, 44, p. 540, 1905.

[2] Buxton and Shaffer, *Journ. Med. Research*, 13, p. 549, 1905. Quoted by Mendel and Leavenworth, *Amer. Journ. physiol.*, 21, p. 95, 1908.

[3] Mendel and Leavenworth, *ibid.*

[4] Cotte, *C. R. Soc. Biol.*, 53, p. 95, 1901.

[5] Mesnil, *Ann. de l'Inst. Pasteur*, 15, p. 352, 1901.

animals seem to have been examined, though doubtless many or all of them contain such an enzyme.

Lipolytic Endoenzymes in Plants.—Upon plants a considerable number of observations have been made, especially within recent years. As long ago as 1876 Schützenberger[1] showed that when an oil-containing seed is pounded in water, an emulsion is obtained which is found after a time to contain free glycerin and fatty acid. He attributed this hydrolysis to the action of an enzyme. The enzyme itself was discovered by Reynolds Green[2] in 1889 in the germinating seeds of *Ricinus*, the castor-oil plant. The endosperms of seeds which had germinated for five days were ground up with 5 per cent. sodium chloride solution containing ·2 per cent. of potassium cyanide, and after standing for twenty-four hours the liquid was filtered. It remained slightly opalescent. Some of the extract was incubated with castor-oil emulsion, and after about half an hour it began to develop acidity owing to the formation of free fatty acid. When allowed to digest for a week in a dialysing tube suspended in distilled water, the reaction of the mixture became more and more acid, whilst the liberated glycerin dialysed out. After concentration of the dialysate, it was detected by means of the acrolein test. This vegetable lipase was found to act best in neutral solution. It also acted well in presence of dilute alkalis, but ·066 per cent. of HCl reduced its activity to a fourth, and ·13 per cent. HCl stopped it almost entirely. Green found that there was no lipase in the resting seeds of Ricinus, but that ground seeds, if kept at 35° for a few hours in the presence of dilute acetic acid, gradually developed their lipolytic power. A saline extract of the resting seeds, faintly acidulated, underwent a similar change, so presumably the acid liberated the enzyme from a zymogen.

The existence of lipase was subsequently demonstrated by Sigmund[3] in both the resting and germinating seeds of rape, hemp, flax, maize, and the opium poppy. The seeds, crushed

[1] Schützenberger, quoted from R. Green's *Soluble Ferments and Fermentation*, 2nd ed., Cambridge, 1901, p. 242.

[2] R. Green, *Proc. Roy. Soc.*, 48, p. 370, 1890.

[3] Sigmund, *Monats. f. Chem. Wien*, 11, p. 272, 1890; *Sitzungsber d. k. Akad. d. Wiss. in Wien*, 99, p. 407, 1890, and 100, p. 328, 1891.

in water, yielded an emulsion which gradually increased in acidity on standing. The lipase also possessed the power of hydrolysing olive oil. Sigmund found that the enzyme could be precipitated from aqueous extracts of bruised mustard and almond seeds by alcohol, and the precipitate, washed with alcohol and dried at 40°, yielded an active solution.

A lipase was prepared by Gérard[1] from the mould *Penicillium*, and by Camus[2] from *Aspergillus niger*. Biffen[3] worked with the mycelium of a fungus which sometimes attacks coconuts during germination. He cultivated it on sterilised coconut milk, and then ground it up with kieselguhr. On filtering under pressure through several thicknesses of filter paper, he obtained an opalescent fluid which not only decomposed monobutyrin, but also coconut oil. The enzyme could be precipitated with alcohol, and the precipitate dried and dissolved up again without loss of activity.

Lipolytic ferments both of animal and vegetable origin have always been found to offer especial difficulties to the investigator, and hence the literature of the subject teems with contradictory statements. As regards the lipase of Ricinus seeds, for instance, Connstein, Hoyer, and Wartenberg[4] found that the activity is most marked in a strongly acid medium, and does not show itself at all in an alkaline medium as Green stated. Taylor[5] obtained an active preparation in the form of a powder from pounded Ricinus seeds which had been extracted with ether, and this preparation hydrolysed the ester triacetin best in a feebly acid medium. H. E. Armstrong[6] also found that Ricinus lipase could hydrolyse only in presence of acid, and that practically any acid is effective. For instance, 1 gm. of fat-free castor-oil seed, kept with 5 c.c. of olive oil and 10 c.c. of 3/100 N sulphuric acid for eighteen hours at 38°, liberated 4·1 gms. of oleic acid. Aspartic and glutamic acids were also very efficient.

[1] Gérard, *Comptes Rendus*, 124, p. 370, 1897.
[2] Camus, *C. R. Soc. Biol.*, 49, pp. 192 and 230, 1897.
[3] Biffen, *Ann. Bot.*, 13, p. 336, 1899.
[4] Connstein, Hoyer, and Wartenberg, *Ber.*, 35, p. 3988, 1902.
[5] Taylor, *Journ. Biol. Chem.*, 2, p. 87, 1907.
[6] H. E. Armstrong, *Proc. Roy. Soc.*, B. 76, p. 606, 1905.

We saw above that Green obtained a filtered extract of Ricinus lipase, only slightly opalescent, which had the power of hydrolysing castor oil. Astrid and Euler[1] likewise found that the filtered juice which had been expressed from rape seed (germinated for sixteen days), hydrolysed ethyl butyrate, but about five times more slowly than the press cake. The enzyme acted best at the natural acidity of the juice. Armstrong, however, was quite unable to obtain an active filtered extract, either of the freshly ground material, or after the extraction of fatty matter or addition of acid. Also Hoyer[2] denies that the lipolytic ferment is soluble in water. Nicloux[3] goes so far as to say that the fat-splitting body of Ricinus seeds is not an enzyme at all. By mechanical means he was able to separate the cytoplasm of pounded Ricinus seeds from all the other cellular elements, and this cytoplasm had a considerable lipolytic power. It acted on fats in the same way as an enzyme, and followed the laws of enyme action. Nevertheless the active substance, which appeared to be attached to the cytoplasm, is not a true enzyme, according to Nicloux, in that it is destroyed by water as soon as it is no longer protected by fats. He calls it lipaseïdine.

From this mass of conflicting evidence it is impossible for the present to pick out the true and the false. We can only await the results of further investigation.

Carbohydrate-splitting Endoenzymes.—We have seen in the two preceding lectures that the tissues contain a series of proteolytic endoenzymes, which effect the gradual degradation of native proteins and nucleoproteins through numerous intermediate stages till they finally split them up into simple bodies such as ammonia, urea, and other products. Similarly, many of the tissues contain a series of carbohydrate-splitting enzymes, which hydrolyse complex polysaccharides like glycogen through intermediate stages of dextrins and maltose to dextrose, and finally, perhaps, split the dextrose still further, with the formation of alcohol and lactic acid, and ultimately of carbon dioxide and water.

[1] Astrid and Euler, *Zeit. f. physiol. Chem.*, 51, p. 244, 1907.

[2] Hoyer, *ibid.*, 50, p. 414, 1907.

[3] Nicloux, *Proc. Roy. Soc.*, B. 77, p. 454, 1906, where further literature is given.

Distribution of Glycogen.—Before discussing the glycogen-hydrolysing enzyme, it is desirable to enquire briefly into the distribution of glycogen in the tissues. As is well known, the liver is generally the richest of all in glycogen, and it sometimes contains as much as all the other body tissues put together. The amount present is extremely variable, the liver of a dog having been found by Schöndorff[1] to contain as much as 18·7 per cent. of it. The muscles come next in their glycogen content, as much as 3·7 per cent. being found by Schöndorff in the muscles of the dog. The percentage varies considerably in different muscles as is shown by the following data, which were obtained by Aldehoff[2] for a twenty-five year old horse which had been starved nine days before being killed.

Tissue.		Per cent. of Glycogen.
Liver		·42
Heart		·82
Muscles	Glutæus maximus	2·44
	Latissimus dorsi	1·29
	Obliquus abdom. ext.	1·71
	Biceps brachii	1·47

The richness of the muscles of this particular animal in glycogen is remarkable, and much above the average of that found in well-fed animals.

Practically all the other tissues of the body contain glycogen. It has been detected in the kidneys, salivary glands, lungs, testes, ovaries, gastric mucous membrane, involuntary muscle, brain, connective tissues and epithelial tissues, not only of vertebrate animals, but also in the corresponding tissues of invertebrate animals.[3] The amounts of glycogen present are generally much smaller than those found in liver and muscle. For instance, Händel[4] found ·03 per cent. in the spinal cord of the ox, and ·10 per cent. in cartilage, ·03 per cent. in tendon, and ·008 per

[1] Schöndorff, *Pflüger's Arch.*, 99, p. 191.
[2] Aldehoff, *Zeit. f. Biol.*, 25, p. 147.
[3] *Cf.* Pflüger, *Pflüger's Arch.*, 96, p. 159, 1903.
[4] Händel, *ibid.*, 92, p. 104.

cent. in bone of the dog. Cramer[1] found ·07 to ·10 per cent. in the human placenta, ·10 to ·19 per cent. in the lungs of new-born children, ·008 to ·018 per cent. in the human brain, ·85 per cent. in the intestine, and ·05 to ·07 per cent. in the skin.

Upon the glycogen content of embryonic tissues erroneous statements have gained wide currency, for their supposed richness in the carbohydrate is unsupported by the results of recent analysis. Microscopical examination is, of course, valueless for quantitative purposes, though it is useful in showing that glycogen is absent from some embryonic organs as the spleen, connective tissues, bones, and nervous tissues, but present in striped muscle and cartilage.[2] Adamoff[3] has made some exact quantitative determinations by Pflüger's method, and he finds that newly hatched chicks contain only traces of glycogen in their bodies. New-born rabbits contain ·17 to ·86 per cent. of glycogen, or less than well-nourished adult animals, whilst the human liver at a late fœtal period contains ·46 to 1·68 per cent. Bernard[4] found no glycogen at all in the liver in early embryonic life, though it appeared towards the middle of intra-uterine development. Pflüger[5] found glycogen in the liver of all the embryos he examined. It was very variable in amount, and sometimes was present only in traces: but the muscles always contained a considerable store. Lochhead and Cramer[6] found that the liver of fœtal rabbits contained very little glycogen up to the twenty-fifth day of gestation, and then it rose above that of the rest of the fœtal tissues. At the same period the glycogen in the maternal placenta, which had previously been considerable, showed a rapid and progressive diminution till the end of gestation. Mendel and Leavenworth[7] estimated the glycogen in pig's embryos, and they found ·25 per cent. of it in the total body tissues of a 50 mm. embryo, ·5 per cent. in a 137 mm. embryo, and a maximum of ·69 per cent. in the

[1] Cramer, *Zeit. f. Biol.*, 24, p. 75, 1887.

[2] Lubarsch, *Arch. f. path. Anat.*, 1906, 183, p. 192.

[3] Adamoff, *Zeit. f. Biol.*, 46, p. 281, 1905.

[4] Bernard, *Journ. de la Physiol.*, 2, p. 326, 1859.

[5] Pflüger, *Pflüger's Arch.*, 95, p. 19, 1903; 102, p. 305, 1904.

[6] Lochhead and Cramer, *Journ. Physiol.*, 35, p. xi., 1906; *Proc. Roy. Soc.*, B. 80, p. 263, 1908.

[7] Mendel and Leavenworth, *Amer. Journ. Physiol.*, 20, p. 117, 1907.

largest embryo examined—one of 212 mm. The liver and brain substance of 85 to 230 mm. embryos contained no glycogen at all, but the combined muscular and skeletal structures contained ·47 per cent. in the smallest embryos, and 1·10 per cent. in the biggest.

Amylases.—Corresponding to this almost universal presence of glycogen, we should expect to find a glycogen-hydrolysing enzyme, which was richest in the liver and muscles. The existence of a diastatic enzyme in the liver was first demonstrated by von Wittich[1] and by Claude Bernard,[2] but subsequent to them, several observers failed to obtain satisfactory evidence of its existence. However, Pavy[3] has demonstrated it by a convenient and quite unexceptionable method. He removed the liver from rabbits directly after death, minced it up finely, pounded it in a mortar, and stirred the pulp with a large volume of absolute alcohol. After standing with the alcohol for as long as six months, he washed it with ether, dried and powdered it. Two grammes of this dry powder were thrown into boiling water to destroy the ferment, whilst another 2 gms. were kept with 20 c.c. of 1 per cent. NaCl solution in an incubator for four hours at 46°. The boiled control was found by titration to contain ·46 per cent. of reducing sugar (on the weight of liver taken), whilst the incubated liver yielded 4·27 per cent. This great increase of sugar must have been due to the action of a diastatic enzyme on the glycogen present in the liver substance. Pavy found that the enzyme was not destroyed even on boiling the liver powder with absolute alcohol.

Though the liver undoubtedly contains a fairly active enzyme, it is doubtful if it is a soluble body to the same extent as the proteolytic endoenzymes described in the previous lectures. It seems to cling somewhat firmly to the liver tissue, and can only be extracted therefrom in small quantities. Miss Eves[4] extracted powdered alcohol-coagulated sheep's liver for forty-eight hours with twice its weight of 10 per cent. sodium chloride solution, and added 2 c.c. of the filtered solution to 10 c.c. of ·5 per cent. starch paste, and another 2 c.c. to 10 c.c. of

[1] von Wittich, *Pflüger's Arch.*, 7, p. 28, 1873.
[2] Bernard, *Comptes Rendus*, 85, p. 519, 1877.
[3] Pavy, *Journ. Physiol.*, 22, p. 391, 1898.
[4] Eves, *ibid.*, 5, p. 342, 1884.

·5 per cent. glycogen solution. After twenty minutes' digestion at 38°, no reduction could be obtained with Fehling's solution, but there was a copious one after an hour. The reducing power of the solutions increased in succeeding hours, but there was still some unaltered starch and glycogen left after twelve hours' incubation. The enzyme could be precipitated from the saline solution by absolute alcohol, and when re-dissolved six days later furnished a weak diastatic solution. Again, Miss Tebb[1] found that the enzyme could be extracted from dried liver powder by means of 5 per cent. sodium sulphate solution. The salt was removed from this solution by dialysis, and on incubating it with 4 per cent. of glycogen at 37° for twenty-one hours, dextrose was formed in some quantity. Such positive evidence as this clearly outweighs the negative results obtained by Noel Paton[2] and other observers.

In that liver tissue after death and disintegration undoubtedly contains an amylolytic enzyme, there can be practically no doubt that such an enzyme exists during life, bound up in the protoplasm of the cells, and exerting its activity whenever it is required. In the light of recent investigation the half-forgotten controversy as to the causation of the post-mortal formation of sugar in the liver is readily set at rest. Noel Paton maintained that in the excised liver there is an early rapid amylolysis preceding, and probably accompanying, the disintegrative changes in the cells, such change being effected by the vital processes of the organ; and that subsequently there is a much slower amylolysis which continues after the disintegration of the liver cells and lasts for many hours, this change being due to the development of an enzyme formed by the disintegration of the cells. Pavy,[3] on the other hand, maintained that the conversion of glycogen into sugar is entirely the work of an enzyme, such enzyme not being present in the cells during life, but only formed on their death from some pre-existent zymogen. If Pavy's hypothesis be accepted for the diastatic enzyme of the liver, then it must be accepted for any and every

[1] Tebb, *Journ. Physiol.*, 22, p. 423, 1898.

[2] Paton, *ibid.*, 22, p. 121, 1897.

[3] Pavy, *The Physiology of the Carbohydrates*, An Epicriticism, 1895, p. 102.

intracellular enzyme, whether proteolytic, lipolytic, or amylolytic. Such an hypothesis, though it cannot for the present be definitely disproved, is sufficiently improbable to carry its own condemnation.

Observations upon the glycogen-hydrolysing enzyme of muscle are almost as numerous as those upon the liver, and just as contradictory. This is largely because of the unsatisfactory methods used by most of the earlier observers for the estimation of glycogen. Almost all the observations concern the rate of disappearance of glycogen from muscles after death. Takácz[1] concluded, from experiments on rabbits, that this disappearance is very rapid, as he found no trace of glycogen thirty minutes after death. Praussnitz[2] found that from the muscles of hens 25 to 59 per cent. of the glycogen disappeared in thirty to sixty minutes. A. Cramer[3] found that muscle separated from the body and kept at 40° showed a considerable diminution of glycogen in four hours, and Seegen[4] obtained a similar result. On the other hand, Boruttau[5] found that only ·2 to 11·1 per cent. of the glycogen disappeared from skeletal muscle in twenty-four to thirty-eight hours, whilst 24 to 100 per cent. disappeared from cardiac muscle in the same time. E. Külz[6] likewise found only a slow disappearance of glycogen from muscle after death, and Böhm[7] even stated that there was no disappearance at all in six to twenty-four hours, provided that putrefaction was prevented.

These observations, taken as a whole, undoubtedly prove the post-mortal disappearance of glycogen from muscle, but they do not definitely show how far this disappearance is the work of an enzyme, and how far dependent on the vital activities of the still living muscle. However, Nasse[8] has demonstrated the existence of an amylolytic enzyme in muscle juice, and

[1] Takácz, *Zeit. f. physiol. Chem.*, 2, p. 372, 1878.
[2] Praussnitz, *Zeit. f. Biol.*, 26, p. 377, 1890.
[3] Cramer, *ibid.*, 24, p. 66, 1888.
[4] Seegen, *Centralb. f. med. Wiss.*, 1887, No. 20 and 21.
[5] Boruttau, *Zeit. f. physiol. Chem.*, 18, p. 513.
[6] Külz, *Pflüger's Arch.*, 24, p. 1, 1881.
[7] Böhm, *ibid.*, 23, p. 44, 1880.
[8] Nasse, *Zur. Anat. u. Physiol. d. quergestreiften Muskelsubstanz*, Leipzig, 1882.

Halliburton[1] found that a watery extract of the dried alcoholic precipitate of muscle juice would change glycogen into a reducing sugar. It had a similar but slower action upon starch, and at a temperature of 40° no sugar was discoverable till the enzyme had acted for five or six hours. Recently Kisch[2] has made an exhaustive research upon the enzyme. He chopped up the muscles of the back and the lower extremities of rabbits a few minutes after death, and mixed 100 gm. of muscle with 100 c.c. of saline, ·5 gm. of glycogen, and toluol, and one to four and a half hours later estimated the glycogen still present by the exact method of Pflüger. In one hour at room temperature, 8 to 68 per cent. of the glycogen disappeared, whilst in parallel experiments in which a current of air was drawn through the tissue pulp, 18 to 75 per cent. disappeared. If a small quantity of oxalate blood were added, there was likewise an increased hydrolysis of glycogen, especially if air were drawn through in addition. The rate of glycolysis was not appreciably influenced by the addition of 20 per cent. of decinormal sulphuric acid or caustic soda. Temperature had a great effect, the amount of glycogen hydrolysed by 100 gm. of muscle in one hour being on an average ·07 gm. at 15°, ·15 gm. at 22°, and ·40 gm. at 36°.

Though the rates of amylolysis were very different in different animals, yet the muscles of each individual gave fairly similar rates with the exception of cardiac muscle. This was much more active than skeletal muscle. For instance, in the case of two rabbits the cardiac muscle hydrolysed 71 and 72 per cent. respectively, and the skeletal muscle 36 and 27 per cent. respectively.

As might be expected from the observations upon the relation of ereptic value to functional capacity described in the last lecture, the enzyme content of a muscle was found to be practically uninfluenced by brief changes in its condition. For instance, the sciatic nerve on one side of a dog was cut, and the remaining muscles were thrown into convulsions for fifty minutes to three hours by means of strychnin injections. Though some of the glycogen had disappeared from the stimulated muscles as

[1] Halliburton, *Journ. Physiol.*, 8, p. 182, 1887.
[2] Kisch, *Hofmeister's Beitr.*, 8, p. 210, 1906.

the result of contraction, they contained no more enzyme than the resting muscles. Again, the muscles of a rabbit which had undergone violent contractions as the result of strychnin injection, were found to contain as much enzyme when tested two hours post mortem as when tested directly after death, and nearly as much when tested five hours post mortem.

Upon tissues other than liver and muscle extremely few observations have been made. Foster[1] observed that extracts of kidney and bladder wall, and also pleural, peritoneal, and pericardial fluids, had a very slight amylolytic action upon starch, whilst extracts of lymphatic glands were inert. von Wittich[2] found small quantities of diastatic ferment in the kidney, brain, and gastric mucous membrane. These observations were made forty years ago, and apparently have not since been repeated and extended, except in one instance. Pick[3] made a single comparison of the digestive powers of minced liver and kidney tissue, and found that in three hours 100 gm. of liver hydrolysed ·69 gm. of glycogen, whilst 100 gm. of kidney hydrolysed 2·39 gm. Hence the kidney tissue is apparently much richer than the liver in the amylolytic enzyme, just as it is richer in β-protease and erepsin. But it is impossible to draw conclusions from a single observation, and until a complete series of comparisons has been made of the amylolytic power of the various tissues, we cannot say definitely whether, and to what extent, the enzyme activity corresponds with the power of the cellular protoplasm to store up and utilise glycogen. At least this is the case as regards the tissues of full-grown animals. Upon embryos a series of observations has recently been made which strongly supports the hypothesis of correlation. Mendel and Saiki[4] minced up the liver and the muscles of pig's embryos of various sizes, allowed the pulp to stand some days under alcohol, and then dried and powdered it. They kept ·5 gm. samples of these powders with 40 c.c. of 1 per cent. glycogen solution and toluol for forty-eight hours at 24°, and determined the amount of sugar formed by gravimetric analysis. The data in

[1] Foster, *Journ. Anat. and Physiol.*, 1, p. 107, 1867.
[2] von Wittich, *Pflüger's Archiv.*, 3, p. 340, 1870.
[3] Pick, *Hofmeister's Beitr.*, 3, p. 174, 1903.
[4] Mendel and Saiki, *Amer. Journ. Physiol.*, 21, p. 64, 1908.

the table show the weights of CuO obtained for each 100 gm. of fresh tissue taken. We see that the reducing power of the liver digests increased very greatly with the growth of the embryos, till in the largest embryos it reached that of the full-grown organ. Muscle had a considerably greater initial amylolytic power, but this increased relatively less rapidly with

Average Length of Embryo.	CuO from Liver digest.	CuO from Muscle digest.
mm.	gm.	gm.
33	·79	1·40
81	·62	1·56
125	1·12	1·58
188	1·46	1·74
225	2·99	3·42
275	5·90	4·93
Adult pig	3·82	...

growth than that of the liver. These results agree well with the previously recorded observations to the effect that the glycogen content of the pig embryo liver is small at first, and increases considerably with growth, whilst that of the muscles is always large, and increases less markedly with growth.

Probably the time and rate of increase of the amylolytic power in the liver and other tissues varies in different animals, for Pugliese[1] found that the blood and liver of new-born dogs and cats contained very little diastatic enzyme, or occasionally none at all, but that it quickly increased in amount with growth of the animal, especially in the case of the liver. Again, Stauber[2] found that an ox embryo 15 cm. in length contained no diastatic ferment in the pancreas, parotid and thymus. On the other hand, the thymus of embryos 23 cm. in length possessed strong diastatic properties, though the brain, lungs, stomach, liver, spleen, kidneys, and muscle were practically free from the ferment. After birth the ferment gradually disappeared from the thymus.

Since the time of Majendie and Claude Bernard it has been

[1] Pugliese, *Arch. d. Farmacologia e Terap.*, 12, p. 1.
[2] Stauber, *Pflüger's Arch.*, 114, p. 619.

known that blood serum can change starch into sugar. Pick[1] found that 100 c.c. of blood digested ·31 gm. of glycogen in three hours, as against the ·69 gm. digested by 100 gm. of liver, hence the small amylolytic activity of the tissues other than liver, muscle, and kidney may be due entirely to the blood enzyme. Bial[2] found that the enzyme could be extracted from the serum of blood and the lymph, but not from blood corpuscles. He stated that the enzyme converted starch into glucose, and so differed from the diastatic enzyme of the salivary gland. Röhmann,[3] and later Hamburger,[4] arguing from the fact that saliva, pancreatic juice, intestinal juice, and blood serum converted starch into maltose and maltose into dextrose at very different relative rates, concluded that they all contain different proportions of two distinct enzymes, viz. a diastase or amylase which converts starch into dextrin and maltose, and a glucase or maltase, which converts these products into glucose. Ascoli and Bonfanti[5] go still further, and think that the blood serum contains several amylases. They find that serum has different rates of action upon different starches, and that it acts more quickly upon a mixture of two starches than upon either starch individually. For instance, human serum, when acting upon 2 per cent. potato starch paste, yielded ·095 per cent. of reducing sugar in a given time; when acting upon 2 per cent. rice starch, ·076 per cent. of sugar, but when acting upon a paste containing 1 per cent. of potato starch and 1 per cent. of rice starch, it gave ·110 per cent. of sugar. In the light of recent knowledge upon the multiplicity of toxins, precipitins, lysins and other bodies in the blood, it seems very probable that Ascoli and Bonfanti are correct in their contention, though many more observations are required before it can be accepted as proven.

But there can at least be no doubt as to the existence of two distinct classes of carbohydrate-splitting enzymes in the blood and tissues, viz., enzymes which hydrolyse polysaccharides like starch, glycogen, and dextrins into maltose or some other

[1] Pick, *Hofmeister's Beitr.*, 3, p. 174, 1903.
[2] Bial, *Pflüger's Arch.*, 52, p. 137, 1892; and 53, p. 156, 1893.
[3] Röhmann, *Ber.*, 27, p. 3251, 1894.
[4] Hamburger, *Pflüger's Arch.*, 60, p. 543, 1895.
[5] Ascoli and Bonfanti, *Zeit. f. physiol. Chem.*, 43, p. 156, 1904.

disaccharide, and enzymes which hydrolyse maltose and other disaccharides into monosaccharides such as glucose. The existence of these two classes of enzymes was suggested by the investigations of Brown and Heron[1] in 1880. Brown and Heron found that aqueous extracts of the minced intestine of the pig had but little action upon starch or cane-sugar, but that if the well washed intestine were dried rapidly in a current of air at 35°, fine shreds of it, added to the starch or sugar under examination, exerted a powerful hydrolytic action. It seemed, in fact, that the endoenzymes clung somewhat firmly to the tissue, and only passed into solution in small quantity.

Maltase.—To test the activity of the dried intestine, Brown and Heron kept 5 gm. of it with 100 c.c. of 3 per cent. soluble starch at 40° for three and a half hours, and then for another sixteen hours at room temperature. Analysis showed that about half of the starch had been converted into sugar, but somewhat unexpectedly, this sugar was found in four experiments out of five to consist entirely of dextrose. In the single experiment in which maltose was present at all, it formed less than a fourth of the total sugar. Further experiment showed that the whole of the starch must have passed through the maltose stage, but that the enzymes of the intestine had a more energetic action upon maltose than upon starch or dextrins, and so directly the starch had been converted into maltose, this maltose was seized upon and broken down further into dextrose. For instance, the shredded dried intestine, if allowed to act upon 3·1 per cent. maltose solution, converted it entirely into dextrose when digested for sixteen hours at 40°. Pancreatic extract, in contradistinction to dried intestine, converted starch rapidly to the maltose stage, and then very slowly converted some of this maltose into dextrose. But malt extract, according to Brown and Heron, had no further action upon the maltose. Following Röhmann's interpretation, we therefore conclude that malt extract contains no trace of maltase enzyme; pancreatic extract contains a small quantity of it, and intestinal extract a large quantity.

Arguing from the presence of maltase in pancreatic extracts, it has been generally assumed that the juice secreted by the

[1] Brown and Heron, *Proc. Roy. Soc.*, 30, p. 393, 1880.

pancreas likewise contains a small amount of maltase, but as far as I am aware, this has never been proved, and it is more probable that the maltase in extracts consists entirely of a soluble endoenzyme, such as is present in most if not all of the other body tissues, and is not secreted into the pancreatic juice as an exoenzyme.

The proof of the general distribution of a maltose-splitting enzyme in the tissues we owe to Miss Tebb.[1] Working on similar lines to Brown and Heron, Miss Tebb dried and shredded various tissues of the pig, and added 5 gm. of each to 100 c.c. of 2·7 per cent. maltose. After twenty hours' digestion at 38°, the following percentages of the maltose were found to have undergone conversion into dextrose :—

Tissue.	Per cent. of Maltose hydrolysed.	Tissue.	Per cent. of Maltose hydrolysed.
Mucous membrane of small intestine . .	76	Kidney . . .	40
Spleen	57	Gastric muc. memb. .	31
Lymphatic gland . .	48	Pancreas . . .	24
Liver	44	Salivary gland . .	17·4
		Skeletal muscle . .	16·7

Intestinal mucous membrane gave the best result of all, whilst the pancreas and salivary glands contained less of the enzyme than any tissue but muscle. A result such as this shows very clearly how completely independent of one another are the powers possessed by a gland of elaborating a diastatic enzyme for secretion externally, and of storing up another kind of carbohydrate-splitting enzyme within its cell substance. The figures also seem to imply that the richness of a tissue in intracellular maltase bears but little relationship to its richness in intracellular amylase, for muscle, which seems to be rich in glycogen-hydrolysing enzyme, is very poor in maltase.

In contrast to Brown and Heron, Miss Tebb found that the maltase readily passed into solution when fresh intestinal mucous membrane was minced and kept in chloroform water. Even the dried intestine, when extracted with 5 per cent. sodium sulphate, yielded an active solution. Extracts of dried lymphatic gland,

[1] Tebb, *Journ. Physiol.*, 15, p. 421, 1894.

pancreas, and liver were likewise moderately active. Probably Brown and Heron did not mince the tissue sufficiently, and they certainly did not allow sufficient time for adequate extraction. This requires several days for aqueous extracts, and three weeks or more for glycerin extracts, whereas they allowed only ten to fifteen hours. There can be no doubt as to the solubility of the enzyme, once it has broken free from its anchorage in the tissues, for Miss Tebb found that serum was far richer in it than any of the organs investigated. Serum diluted with three volumes of water converted the whole of the 2·7 per cent. of maltose added to it into dextrose in twenty-three hours at 38°. Assuming that serum contains 8 per cent. of solids, it follows that only 2 gm. of solid serum were used for each 100 c.c. of maltose, as against 5 gm. of solids in the case of the tissues above mentioned. This richness of serum in maltase introduces a disturbing factor into the above recorded maltase values of the tissues, for they were obtained with organs from which the blood and lymph had not been removed, and so an unknown fraction of their apparent maltose-splitting power is due to retained serum. A repetition of the observations with previously perfused organs is therefore desirable.

Upon the maltase content of embryonic tissues no quantitative observations have been made. Bierry[1] found the enzyme in the intestines of embryonic sheep and cattle, whilst Mendel and Mitchell[2] found that it was present in the intestines of all pigs' embryos over 50 mm. in length. A single observation on the kidneys of a 120 mm. embryo showed no maltase, whilst one on the liver of a 200 mm. embryo showed a trace of it.

Invertase.—Though there is some doubt as to the existence of more than one amylolytic enzyme in the tissues, there are certainly at least three distinct enzymes which act upon disaccharides, viz. maltase, invertase, and lactase. Invertase, which has the power of hydrolysing cane-sugar to glucose and fructose, was isolated by Berthelot[3] from yeast in 1860. Subsequently Claude Bernard showed that an infusion of intestinal mucous membrane likewise contained the enzyme. He demonstrated its

[1] Bierry, *C. R. Soc. Biol.*, 52, p. 1080.
[2] Mendel and Mitchell, *Amer. Journ. Physiol.*, 20, p. 81, 1907.
[3] Berthelot, *Comptes Rendus*, 50, p. 980, 1860.

presence in the intestine of dogs, rabbits, birds, and frogs.[1] Other observers found it in the intestinal tract of man, the horse, ox, and cat. It is also present in succus entericus, but Röhmann[2] found that extracts of the mucous membrane are much richer in enzyme than the secretion, so probably it is chiefly intracellular in its action, and splits up the cane-sugar during its passage through the intestinal wall. Röhmann,[3] and also Miura,[4] found that the upper part of the small intestine contains more invertase than the lower part, and Miura found that the enzyme is also present in small amount in the tissues of the colon, stomach, and pancreas. However, Harris and Gow[5] failed to find it in the pancreas, and Widdicombe[6] found none in lymphatic glands. Hence it is not an enzyme of widespread distribution like maltase, but is practically limited to the mucous membrane of the alimentary canal.

The inverting ferment of the small intestine is much less active than the maltase. Brown and Heron found that in two parallel experiments 27 and 24 per cent. respectively of the cane-sugar was split up, but 74 and 58 per cent. respectively of the maltose. Widdicombe observed an interesting relationship of the enzyme to the reaction of the medium in which it was digesting. He found that in ·3 to ·5 per cent. HCl the enzyme of intestinal mucous membrane was inactive, though the inverting action was merely suspended under the influence of the acid, not destroyed. Gastric mucous membrane, on the other hand, and likewise gastric juice, inverted cane-sugar readily in an acid medium, but not in an alkaline one.

Lactase.—The distribution of lactase in animal tissues is even more limited than that of invertase, for it is confined entirely to the intestine, and in some cases to the intestine of young animals only. It was discovered by Röhmann and Lappe[7] in the mucous membrane of the small intestine of dogs and calves.

[1] Bernard, *cf.* R. Green, *Soluble Ferments and Fermentation*, p. 115, 1901.

[2] Röhmann, *Internat. Physiol. Kongr., Turin*, 1901.

[3] Röhmann, *Pflüger's Arch.*, 41, p. 411, 1887.

[4] Miura, *Zeit. f. Biol.*, 32, p. 266, 1895.

[5] Harris and Gow, *Journ. Physiol.*, 13, p. 469, 1892.

[6] Widdicombe, *ibid.*, 28, p. 175, 1902.

[7] Röhmann and Lappe, *Ber.*, 28, p. 2506, 1895.

Aqueous extracts slowly split up lactose into glucose and galactose. The enzyme could be precipitated from the extracts by alcohol, and a solution of the precipitate possessed the power of hydrolysing lactose. In all probability lactase is purely an endoenzyme, as Pregl[1] found that the intestinal juice of the lamb, collected from a Thiry-Vella fistula, contained none of it. Fischer and Niebel[2] found that intestinal extracts made from young animals were frequently more active than those from old ones. Portier[3] found lactase in the intestine of old rabbits, but not in that of pigs and birds. Weinland[4] examined the intestine of young and old animals, and found that lactase was always present in young animals, and also in old dogs, pigs, and horses, but not in the intestine of old oxen, sheep, rabbits, and fowls. As a rule the amount of lactase present is small, and its detection by no means easy, so the results obtained cannot be accepted implicitly. However, Aders Plimmer[5] has recently repeated these observations, and using an accurate gravimetric method for estimating the reducing sugar formed, has in the main confirmed them. He ground up the mucous membrane with sand, and treated it for six to twenty-four hours with toluol water. The extract was then filtered through lint, and so still contained cells and cell fragments. Its action was only slow at best. For instance, 150 c.c. of extract of cat's intestine, kept with 150 c.c. of 5 per cent. lactose solution and toluol at 37°, hydrolysed 13 per cent. of the lactose in twenty-six hours, 23·4 per cent. in seventy-two hours, and 46·5 per cent. in 170 hours. Plimmer found that the omnivorous cat and pig have lactase in their intestine during the whole of their lives. The herbivorous guineapig has it only when it is young, but in that the adult rabbit has plenty of lactase, there can be no hard and fast distinction between the two classes of animals. Probably, also, one is not justified in drawing conclusions from a limited number of observations, for Orbán,[6] just

[1] Pregl, *Pflüger's Arch.*, 61, p. 359, 1895.
[2] Fischer and Niebel, *Sitzber. Akad. Wiss. Berlin*, p. 73, 1896.
[3] Portier, *C. R. Soc. Biol.*, 52, p. 423, 1900; 53, p. 810, 1901.
[4] Weinland, *Zeit. f. Biol.*, 38, p. 16, 1899.
[5] Plimmer, *Journ. Physiol.*, 35, p. 20, 1906.
[6] Orbán, *Maly's Jahresb.*, 1899, p. 384.

like Weinland, was unable to find lactase in full-grown rabbits. Plimmer found that neither the frog nor the fowl had lactase in their intestines, so probably the enzyme is confined to mammalia.

Guineapigs one to three days old were found to be rich in lastase, whilst animals five or more weeks old had practically none. Hence the ferment must have dwindled down within these weeks, more or less synchronously with the change of diet from milk to vegetable food. The interesting and important question arises as to whether this is a case of direct adaptation. Plimmer endeavoured to solve it by feeding adult guineapigs upon a milk and lactose diet for five to eleven weeks, but in no case did any appreciable amount of lactase appear in their intestines. One might almost expect such a result as this, for once the tissues have given up the elaboration of any particular enzyme, the mechanism for such elaboration probably disappears, and cannot be acquired again at any rate in a short time. Quite otherwise is the result one would expect if young animals, still possessing lactase-forming powers, were kept permanently on a milk diet. Plimmer records no experiments of this kind, but he says that adult rats and rabbits, when fed on milk and lactose, show no increase in the normal lactase-content of their intestines. However, his experimental data do not altogether bear out this contention. For instance, two rabbits, kept three and fifteen weeks respectively without milk, gave 11·2 and 55·1 per cent. of hydrolysis of the standard lactose solution, whilst two other rabbits, kept for similar periods on a milk and lactose diet, gave 23·4 and 62·3 per cent. of hydrolysis. Again, Sisto[1] obtained results which accord with expectation, for he found that though lactase is present only in small quantities in the intestine of adult mammals, or is not present at all, it appears on continued feeding with milk or milk sugar.

As regards the variation of lactase with growth, Plimmer found that rat embryos had none of the enzyme in their alimentary canal forty-eight hours before birth, whereas it was present twelve hours before birth. On the other hand, Mendel and Mitchell[2] found it in all pigs' embryos more than 50 mm. in length. Probably the lactase attains a maximum within a

[1] Sisto, *Arch. d. Fisiol.*, 4, p. 116.

[2] Mendel and Mitchell, *Amer. Journ. Physiol.*, 20, p. 81, 1907.

very few days after birth, and then dwindles down again. Comparative observations upon invertase do not seem to have been made, but Cohnheim[1] states that it is present in the intestine of fœtal and new-born cats and dogs. In fact it might be the only demonstrable ferment in the fœtal animals. On the other hand Mendel and Mitchell found that it was uniformly lacking from the intestines of pigs' embryos.

Intracellular diastatic enzymes are probably as widely distributed in the lower animals as in the higher. Miss Greenwood[2] found that Rhizopods do not attack starch, but Meissner found that Infusoria, if deprived of protein food, will take it up and dissolve it. De Bary states that starch grains ingested by the plasmodium of Mycetozoa are strongly corroded in the course of some days, whilst Lister concludes that certain of the Mycetozoa have little or no action upon raw starch, but speedily digest swollen starch. Hartog and Dixon found that the large protozoon *Pelomyxa palustris* contained a diastatic enzyme which quickly converted starch into erythrodextrin, but only slowly transformed this body into sugar. In sponges Krukenberg found that diastatic ferments capable of converting starch into sugar are widely distributed, whilst Mesnil found a weak ferment in aqueous extracts of the pulped mesentery filaments of Actinia.

Amylases in Plants.—In plants, diastatic and sucroclastic enzymes are much more widely distributed than in animals, and by reason of their great activity and of their economic importance they have been the subject of a much more thorough study. It is unnecessary to describe them in detail, however, as they have been dealt with fully elsewhere.[3] It will be sufficient to refer to them chiefly in relation to the intracellular diastatic and sugar-splitting enzymes of animal tissues.

The diastase of germinating barley, discovered by Kirchoff in 1814, is the first of the soluble ferments known. Payen and

[1] Cohnheim, *Nagel's Handbuch d. Physiol.*, 2, p. 599, 1907.

[2] Greenwood. See v. Fürth's *Vergleichende Chem. physiol. d. niederen Tiere*, for this and other references, and for fuller details.

[3] See especially Reynolds Green's *Soluble Ferments and Fermentation*, Cambridge, 1901, chaps. ii. and iv.-x., from which this brief summary is mainly drawn.

Persoz found the same ferment in germinating wheat, oats, maize, and rice, and in 1874 Gorup-Besanez found it in other varieties of germinating seeds. Kosmann and Krauch demonstrated it in the leaves and shoots of the higher plants, and in various algæ, lichens, mosses, and fungi. Baranetzky found it in buds and in potato tubers, and in the light of what was known of its distribution, he suggested that it is present in all vegetable cells. Subsequent investigations have tended to confirm this hypothesis, and they have established the additional fact of the existence of at least two different vegetable diastases, viz. the so-called diastase of translocation, and diastase of secretion. The first is the more widely distributed, as it occurs in the seed during the development of the embryo, as well as being present in the vegetative organs. It is most readily prepared from barley, and it differs from secretion diastase in that it dissolves starch grains without corrosion: acts very slowly on starch paste, but quickly on soluble starch: works best at 45° to 50°, and is much more active at a low temperature than secretion diastase. Secretion diastase, on the other hand, is especially connected with the process of germination, and is most readily prepared from malt extract. It corrodes starch grains and disintegrates them before solution: acts rapidly upon starch paste: works best at 50° to 55°, and can be heated to 70° without destruction. Arguing from the fact that vegetable proteins differ from animal proteins, and that enzymes are probably bodies related to proteins, it follows that vegetable diastases cannot be chemically identical with animal diastase. The course of action of the two enzymes is likewise very different,[1] but the final product of activity is in both cases maltose. Probably no isomaltose, dextrose, or other sugar is formed at the same time, and it seems likely that glycogen is also converted by diastase into maltose only.

In addition to diastase, vegetable tissues contain at least two other amylolytic enzymes, which seem to have no analogues in the animal kingdom. Certain of the Compositæ, such as the genera Dahlia and Helianthus, possess tubers or fleshy roots in which stores of the carbohydrate inulin are situated, whilst the bulbous Liliaceæ and related plants contain stores of it in their

[1] *Cf.* Vernon, *Journ. Physiol.*, 28, p. 156, 1902; see also Lect. VI., p. 159.

leaves and elsewhere. This inulin is usually in solution in the sap of cells. In the process of growth of the plants it is transformed into fructose by the action of an enzyme *inulase* (Reynolds Green).[1] Again, the walls of many vegetable cells consist partly of cellulose, pectose, and related substances, the hydrolysis of which is effected by the enzyme *cytase*.

Sucroclastic Enzymes.—In sucroclastic enzymes, as in amylolytic ones, plants are likewise richer than animals, for in addition to maltase, invertase, and lactase, they contain at least three others, viz. *trehalase*, *raffinase*, and *melizitase*. Maltase was first shown to exist in the vegetable kingdom by Bourquelot, who ground up the mycelia of the moulds *Aspergillus niger* and *Penicillium glaucum* with sand, extracted with water, precipitated the maltase in the aqueous extract with alcohol, and on redissolving this precipitate in water obtained an active preparation. Bourquelot also detected maltase in yeast, whilst Cuisinier found it in barley malt, and Geduld in maize.

Invertase is more widely distributed in plants than maltase, for Berthelot discovered it in yeast (1860); Béchamp found it in moulds, and in the petals of several flowers; Brown and Morris found it in the air-dried leaves of *Tropoeolum*, and Kosmann in the buds and leaves of young trees. It is also present in the rootlets of germinated barley. Probably cane-sugar and invertase play an important part in the nutrition of actively growing vegetable protoplasm.

In plant tissues, as in those of animals, lactase seems to be of very restricted distribution. Beyerinck found it in extracts of the *Kephir* organism, whilst Fischer found it in certain yeasts.

Of the remaining three sucroclastic enzymes, *trehalase* is found in certain moulds such as *Aspergillus niger* and *Penicillium glaucum*, where its function is to hydrolyse the disaccharide trehalose into glucose. *Raffinase* is found in the root of the beet, in germinating barley and wheat, and elsewhere. It acts upon the hexatriose body raffinose, $C_{18}H_{32}O_{16}$, and splits it up into glucose, fructose, and galactose. *Melizitase* is found in the manna exuded from the leaves and branches of the leguminous plant, *Alhagi maurorum*, and elsewhere, and it

[1] R. Green, *Ann. Bot.*, 1, p. 223, 1888.

hydrolyses the melizitose therein contained to glucose. Melizitose is a hexatriose like raffinose.

In some plants still another class of sugar-splitting enzymes is represented, viz. the glucoside-splitting enzymes. These enzymes have the property of breaking down various complex bodies with the formation of a sugar, generally glucose, and other products. The best known of them, *emulsin*, has the power of decomposing the glucoside amygdalin into benzoic aldehyde, hydrocyanic acid, and glucose. It will also effect the hydrolysis of other glucosides such as salicin and phlorizin. Emulsin is found in the young stems and leaves of the Cherry-Laurel, in certain Lichens, and in many fungi, as well as in the seeds of sweet and bitter almonds. Another enzyme, *myrosin*, is widely distributed throughout the Cruciferæ, and in several allied Natural Orders, and moreover it is present in the roots, stems, leaves, flowers, and seeds of many of these plants. It splits up the glucoside sinigrin into allyl sulphocyanate, potassium hydrogen sulphate, and glucose.

It is unnecessary to refer to the other glucoside-splitting enzymes which have been described in various plant tissues. Of greater interest to us is the occurrence of similar ferments in animal tissues. Kölliker and Müller[1] showed that pancreatic juice is capable of decomposing amygdalin, but they did not isolate the specific enzyme. Recently Gonnermann[2] has made a number of observations upon the hydrolytic power of various animal tissues upon glucosides and alkaloids. He found that fresh minced liver of the ox and hare were able to decompose the glucosides amygdalin, arbutin, and sapotoxin, but that the liver of the dog, horse, and fish had no action on amygdalin, a feeble one on sapotoxin, and a well marked one on arbutin. Trypsin, or presumably a pancreatic extract, was said to split up amygdalin, but not the other two glucosides, whilst pepsin acted on none of them. The glucoside sinigrin was not attacked by any of the tissues or enzymes investigated. This capacity of animal tissues to split up glucosides seems to imply that a specific enzyme is not required for the process, but that one of the enzymes normally present, perhaps maltase, is able to effect

[1] Kölliker and Müller, see R. Green's *Soluble Ferments*, p. 154, 1901.

[2] Gonnermann, *Pflüger's Arch.*, 113, p. 168, 1906.

it. But even then it is difficult to account for the results obtained by Gonnermann, as one would expect that amygdalin, for instance, if attacked by the liver tissue of one animal, would be similarly decomposed by that of other animals. Before any definite conclusion can be adopted, therefore, a good deal more investigation is necessary.

LECTURE IV

ZYMASE AND OTHER GLYCOLYTIC ENZYMES

Zymase of yeast. Its action on various sugars. Cause of its instability. Effect of filtration. Action of antiseptics. Influence of phosphates. Zymase and lactacidase enzymes. Action of inorganic catalysts on glucose. Glycolytic power of mixed pancreas and muscle juice. Alcohol in animal tissues. Anaërobic respiration in living and dead plants. Formation of acids in aseptic and antiseptic autolyses.

IN the last lecture we were able to trace the endoenzymes which can convert the carbohydrates of the tissues and food into disaccharides, and subsequently into the simpler monosaccharides. But of the further changes which these monosaccharides undergo we know very little with certainty. Until recent years we had no conception of what happened to them, other than that they were oxidised in the tissues to carbonic acid and water. But in the light of recent investigation it seems possible, even probable, that they undergo the same kind of changes as those which have long been known to occur in alcoholic and lactic acid fermentations.

Alcoholic fermentation[1] was first proved to be the work of a definite organism by Cagniard de Latour and by Schwann in 1837. Both these observers thought that the yeast cells formed alcohol and carbon dioxide by their vital processes, and though this view was combated by Liebig, it was supported by the remarkable researches of Pasteur. The recognition of substances which though not composed of living organisms were derived

[1] For a detailed account of the history of alcoholic fermentation, see Reynolds Green, *Soluble Ferments and Fermentation*, Cambridge, 2nd ed., 1901.

from such organisms, and which were able to induce somewhat similar changes to those effected by yeast, led to a separation of fermentations into two classes, viz., those produced by organised ferments such as yeast and putrefactive bacteria, and those produced by unorganised ferments. Many attempts have been made in the past by Naegeli, Sachs, and other investigators, to show that these ferments are radically different, but with increase of knowledge the supposed differences and distinctions were found to dwindle down more and more, till the discovery of zymase by Buchner[1] in 1897 indicated that they could no longer be supported. The further they are analysed the more have the activities of the micro-organisms of fermentation been shown to depend upon intra- or extra-cellular enzymes, and hence it would seem to be only a question of time before they will all of them be referred to this source.

The method used by Eduard Buchner for isolating an enzyme which could decompose sugar into alcohol and carbon dioxide is as follows. Washed yeast is subjected to a pressure of fifty atmospheres in an hydraulic press, whereby 70 per cent. of the water contained in it is squeezed out, and a fine dry white powder left. This powder is mixed with quartz sand and the siliceous earth kieselguhr in the proportions of 10 of yeast, 10 of sand, and 2 or 3 of kieselguhr, and the mixture ground up in a porcelain mortar by means of a very heavy (8 kg.) iron pestle. The yeast cells are broken up by the sharp sand grains, and in a few minutes the dry powder becomes converted into a grey-brown plastic mass, which sticks together in the form of balls. The mass is wrapped up in a strong "press-cloth," and subjected to a gradually increasing pressure by means of a hydraulic press. One kilogram of yeast, subjected to 300 atmospheres pressure, yields as much as 450 to 500 c.c. of juice. In some cases the press cake was taken out of the press and ground up with 100 c.c. of water and again subjected to pressure, when 100 to 150 c.c. more juice was obtained.

Macfadyen, Morris, and Rowland,[2] who have repeated many

[1] Most of the experimental details recorded in the next few pages are taken from *Die Zymasegährung*, by E. Buchner, H. Buchner, and M. Hahn: Munich and Berlin, 1903, pp. 1-416.

[2] Macfadyen, Morris, and Rowland, *Proc. Roy. Soc.*, 67, p. 250, 1900.

of Buchner's experiments in this country, adopted a more thorough method of disintegrating the yeast cells. They mixed the yeast with silver sand and placed it in a vessel in which a many-toothed spindle was rapidly rotated by mechanical power. The sand grains and yeast cells were driven violently against one another, and a microscopical examination at the end of the process showed that every cell was ruptured. Brine at a temperature of $-5°$ was circulated round the vessel, and this kept the disintegrating mass at about 15°.

The press juice, after filtration, was obtained as a yellowish, slightly opalescent liquid. It had a specific gravity of 1·031 to 1·057, and contained 8·6 to 13·9 per cent. of solids, of which 1·3 to 1·9 per cent. was ash, and the remainder mostly protein. On boiling, this coagulated and yielded a solid white mass. In addition to zymase, the juice contained the enzymes invertase, maltase, endotryptase, rennin, a glycogen-hydrolysing enzyme, and a reducing enzyme which could liberate sulphuretted hydrogen from sodium thiosulphate and from sulphur, and decolorise methylene blue. If sugar were added to this juice, carbon dioxide began to bubble off in a few minutes, and a steady stream was evolved for hours or days, according to the temperature. At 35° the outflow was very rapid, but almost ceased after a day, whilst at 6° its initial rate of evolution was six times slower, but it continued with undiminished vigour for ten days or more. At 22° a mixture of 20 c.c. of juice with 8 gm. of cane-sugar, gave it off at the following rate :—

1st day	1·00 gm.
2nd „	·36 „
3rd „	·04 „
4th „	·01 „

Together with this carbon dioxide, alcohol is formed in almost equal quantity, in accordance with the equation :

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O.$$

This requires the formation of twenty-three parts by weight of alcohol for each twenty-two parts of CO_2. One of the most active juices obtained yielded 12·2 gm. of CO_2 and 14·4 gm. of alcohol per 100 c.c. of juice ; another, 12·2 gm. of CO_2 and

12·4 gm. of alcohol; another, 8·9 gm. of CO_2 and 8·9 gm. of alcohol.

Numerous experiments were made by Buchner and Rapp[1] upon the fermentability of various sugars, and from the data given in the table we see that glucose and fructose gave the

20 C.C. OF JUICE + 13 PER CENT. OF SUGAR, KEPT 40 HOURS AT 15°, YIELDED :—

	In presence of ·2 c.c. of Toluol.	Another Sample of Juice, without Antiseptic.
	gm.	gm.
Glucose . . .	·70	·72
Fructose . . .	·70	·73
Galactose . . .	·12	·13
Saccharose . . .	·66	·72
Maltose . . .	·69	·72
Lactose . . .	·02	·03
Glycogen . . .	·29	·23

best yield of CO_2. Cane-sugar and maltose were only slightly inferior, in spite of the fact that they had to be converted by the invertase and maltase enzymes of the juice into the monosaccharide form before they could be acted on by the zymase. On the other hand, galactose was attacked but slightly by the zymase, and lactose practically not at all. Glycogen was acted on somewhat slowly, presumably because the glycogen-splitting enzyme of the juice is only a weak one. Potato starch was even less readily attacked, as a mixture of 20 c.c. of juice (a different sample from the above) with 1 gm. of starch and ·2 c.c. of toluol yielded only ·10 gm. of CO_2 in sixty-four hours. Four grammes of soluble starch, acted on under similar conditions, gave ·13 to ·28 gm. of CO_2, whilst dextrin gave ·57 to ·75 gm. of CO_2.

Buchner found that the greatest yield of CO_2 is obtained by using concentrated sugar solutions containing 30 to 40 per cent. of sugar. (See table on page 85.) The sugar protects the juice against auto-destruction, and so the evolution of CO_2 continues longer with greater concentrations than with smaller ones; but the initial rate of evolution is less. For instance, in the first six hours of the experiment, the 10 per cent. sugar

[1] Buchner and Rapp, *Ber.*, 31, pp. 1084 and 1090, 1898.

mixture gave out ·17 gm. of CO_2, but the 40 per cent. mixture only ·105 gm. Macfadyen, Morris, and Rowland found that their juice (which was made from top fermentation yeast, not bottom yeast like Buchner's) gave the best yield of CO_2 with 5 to 10 per cent. cane-sugar. Also it gave a considerable

20 C.C. JUICE + CANE-SUGAR + ·2 C.C. TOLUOL, KEPT 96 HOURS AT 22° TO 25°, YIELDED :—

Sugar.	CO_2.
Per cent.	gm.
10	·56
15	·64
20	·73
25	·79
30	·81
40	·82

yield when no sugar whatever was added, so presumably it was richer in glycogen than Buchner's juice, which under such conditions evolved scarcely any CO_2.

The properties of zymase have been described thus far without any comment upon their resemblance to or difference from those of enzymes in general on the one hand, and of living yeast cells on the other. In fact, they have been tacitly assumed to be those of an enzyme, though such an assumption is not justifiable without further explanation. This is especially the case in regard to the extreme instability of zymase. This and other curious properties for a time led some investigators to believe that zymase consisted of fragments of still living protoplasm, which, for the short time they remained alive, were able to exert the functions normally confined within the living cell. In the light of more complete knowledge, however, this hypothesis has been almost entirely discarded, and for reasons which are given in detail below, we may now regard the enzymic nature of zymase as undoubtedly established.

The property of extreme instability which seemed more especially to differentiate zymase from other enzymes is dependent in large measure upon the powerful proteolytic enzyme always present in yeast juice. This enzyme, called endotryptase, has been subjected to a detailed study by Hahn

and Geret.[1] It is similar to the β-protease of animal tissues, in that it acts best in feebly acid solutions—the optimum being ·2 per cent. HCl—whilst its activity is paralysed by alkalis. It digests native proteins somewhat slowly, as fibrin flakes require about twenty-four hours for solution by yeast juice, but it rapidly converts albumoses and peptones into amino acids. The coagulable protein present in yeast juice is speedily digested by it to the non-coagulable stage, as the following data show :—

	Coagulable Protein.	N. in Coagulum.	N. in Filtrate.
	Per cent.	Per cent.	Per cent.
Fresh juice.	4·38	·64	·42
After 1 day at room temperature . .	1·43	·19	·85
„ 6 days „ „ . .	·14	·02	1·03

Hahn and Geret also estimated the phosphates in the filtrate from juice which had been precipitated by $HgCl_2 + HCl$, and they observed a very rapid passage of organically bound phosphorus into the soluble inorganic state. Thus of the total ·528 per cent. of P_2O_5 present they found that :—

P_2O_5 in fresh filtrate = ·024 per cent.
P_2O_5 after 12 hours = ·360 „
P_2O_5 after 5 days = ·416 „

In another experiment the filtrate from the fresh juice contained ·020 per cent. of P_2O_5, and after seventy minutes' autodigestion at 39°, no less than ·384 per cent. After nine days it was ·506 per cent., the total P_2O_5 present being ·600 per cent.

Similar evidence of the rapid autodigestion of yeast juice has been obtained by Macfadyen, Morris, and Rowland, by Petruschewsky[2] and others. The endotryptase, when digesting the coagulable protein, doubtless digests the zymase at the same time, so if it were possible to separate the two enzymes it might be found that zymase by itself is no more unstable than other enzymes. No attempts at such separation appear to have been

[1] Hahn and Geret, *Zeit. f. Biol.*, 40, p. 117 ; also, *Die Zymasegährung*, p. 29 *et seq.*

[2] Petruschewsky, *Zeit. f. physiol. Chem.*, 50, p. 251, 1907.

made, though there is no reason why a partial separation should not be effected by fractional precipitation with alcohol or ammonium sulphate solution. However, it is improbable that the endotryptase would be completely removed in this way. Perhaps a better method of neutralising its digestive action upon the zymase would be by means of an antiferment.

The destructive action of the endotryptase upon the zymase can be diminished or increased in a number of different ways. The protective action of concentrated cane-sugar solutions has already been commented on. Glycerin also exerts some influence, and small quantities of alcohol retard the action of endotryptase more than that of zymase. Probably proteins act best of all, in that a good deal of the endotryptase becomes bound to the added protein, and exerts its digestive powers upon it, whilst the zymase is correspondingly spared. A few data proving this point will be quoted below in another connection.

It is much easier to hasten the rate of digestion of the zymase, and so its rate of destruction, than to retard it. The effect of adding another proteolytic enzyme is well shown by the following data:—

20 c.c. Juice + 8 gm. Cane-Sugar + 2 c.c. Toluol, in 96 hours at 22°, gave	·95 gm. CO_2.
20 c.c. Juice + 8 gm. Cane-Sugar + 2 c.c. Toluol + ·4 gm. Trypsin, in 96 hours at 22°, gave . . .	·11 „
20 c.c. Juice + 8 gm. Cane-Sugar + 2 c.c. Toluol + ·4 gm. Pancreatin, in 96 hours at 22°, gave . . .	·05 „
20 c.c. Juice + 8 gm. Cane-Sugar + 2 c.c. Toluol + ·4 gm. Diastase, in 96 hours at 22°, gave . . .	·83 „

Trypsin and pancreatin reduced the CO_2 output to a tenth or twentieth its normal amount, whilst a non-proteolytic enzyme like diastase depressed it but slightly.

The readiest method of increasing the rate of destruction of the zymase is to dilute the press juice. For instance, the dilution of a sample of juice with an equal volume of water reduced its yield of CO_2 from ·46 gm. to ·33 gm. Probably such small dilution acts chiefly by rendering the conditions of action of the endotryptase upon the zymase more favourable. When the dilution was made with a solution of cane-sugar (9 to 29

per cent.) instead of with water, there was practically no diminution of CO_2 output. This is presumably due to the sugar forming a loose combination with the zymase, and so protecting it from the endotryptase. However, the activity of the zymase is very greatly reduced by considerable dilution, as the following data show:—

20 c.c. Juice + 5 or 10 gm. Cane-Sugar + Thymol, in 96 hours at 22°, gave	·97 gm. CO_2
20 c.c. Juice + 5 or 10 gm. Cane-Sugar + 500 c.c. Saline, in 96 hours at 22°, gave	·032 „
20 c.c. Juice + 500 c.c. of 10 per cent. Glycerin containing 1 per cent. Cane-Sugar, in 96 hours at 22°, gave .	·08 „
20 c.c. Juice + 500 c.c. of 10 per cent. Egg White containing 1 per cent. Cane-Sugar, gave	·28 „

The saline used contained K_2HPO_4, $MgSO_4$, $CaSO_4$, NaCl, and 1 per cent. of cane-sugar. It will be seen that 10 per cent. glycerin solution had a slight protective effect upon the zymase, whilst 10 per cent. egg white had a more considerable one. In another experiment a mixture of juice, sugar, and thymol with 500 c.c. of solution containing 10 per cent. of egg white and 2 per cent. of cane-sugar, yielded no less than ·88 gm. of CO_2, as against the 1·20 gm. yielded by the control. The protective influence of proteins referred to above is strikingly shown by these two experiments.

The influence of dilution varies greatly with different samples of press juice, for Wroblewski[1] found that the evolution of CO_2 practically stopped on tenfold dilution. Macfadyen, Morris, and Rowland[2] observed a much greater influence still, for their juice sometimes ceased to evolve CO_2 on dilution with twice its volume of water. Saline solution (·75 per cent. NaCl) acted even more unfavourably, as the addition of an equal volume of it to the juice almost stopped the CO_2 output. Presumably the juice contained a more active endotryptase than Buchner's, for its initial content of zymase—as judged by the CO_2 formation of the undiluted juice—was often as great.

The influence of filtration upon the activity of yeast juice has been the subject of much experiment and discussion, as the

[1] Wroblewski, *Centralb. f. Physiol.*, 13, p. 284, 1899.
[2] *Loc. cit.*

results are somewhat contradictory. Buchner found that the juice could be filtered through a Berkefeld kieselguhr filter without losing much of its activity, *e.g.*, a reduction of CO_2-forming power from 2·08 gm. to 1·62 gm. The pores of the filter are sufficiently small to stop the passage of yeast cells, but not of bacteria, hence it seemed possible that small fragments of living protoplasm might be forced through. Filtration through a Chamberland biscuit porcelain filter, which is said to stop the passage of the smallest bacteria, reduced the activity of the juice in some cases to vanishing point. But Buchner obtained more favourable results by first of all filtering the juice through a kieselguhr filter, whereby the larger particles of cell membrane, protoplasm, and intact yeast cells were removed, and then forcing it through the porcelain filter. The pores of this filter were not so rapidly clogged up as before, and the first portions of juice coming through had about half the activity of the unfiltered juice: but the subsequent portions contained less and less zymase, as the following data show:—

In 88 hours at 16°, 20 c.c. Juice (unfiltered)+8 gm. Sugar gave	·63 gm. CO_2.
First 20 c.c. Juice (filtered)+8 gm. Sugar, in 88 hours at 16°, gave	·30 „
Next 20 c.c. Juice (filtered)+8 gm. Sugar, in 88 hours at 16°, gave	·12 „
Next 20 c.c. Juice (filtered)+8 gm. Sugar, in 88 hours at 16°, gave	·08 „
Next 20 c.c. Juice (filtered)+8 gm. Sugar, in 88 hours at 16°, gave	·08 „

This considerable and increasing reduction in the activity of the filtered juice is easily explained in the light of C. J. Martin's[1] observations on gelatin filters. Martin found that if the pores of a Pasteur-Chamberland candle filter were filled with a 10 per cent. gelatin solution, they are rendered impermeable to colloids such as proteins and starch, but readily permit the passage of crystalloids. Filtration of serum or egg white diluted with an equal bulk of salt solution gave a clear colourless solution absolutely free from protein. Harden and Young[2] found that yeast juice similarly gives an enzyme-free—so presumably

[1] C. J. Martin, *Journ. Physiol.*, 20, p. 364, 1896.

[2] Harden and Young, *Proc. Roy. Soc.*, 77 B, p. 405, 1906.

protein-free—filtrate. In Buchner's experiments the pores of the filter must have got gradually clogged up with protein, till they finally became almost as impermeable as those of the gelatin filter.

Zymase, though so unstable in solution, is almost if not quite as stable as other enzymes when dried. Buchner evaporated the juice *in vacuuo* at 20° to 25°, and in about half an hour it reached a syrupy condition. He then spread it in a thin layer on glass plates, and dried it *in vacuuo* or in air at 35°. After twenty-four hours he powdered it and dried it still more thoroughly by keeping it over sulphuric acid *in vacuuo*. The dry powder was almost completely soluble in water, and had lost scarcely any of its initial activity. For instance, a sample of fresh juice, mixed with sugar, yielded 1·28 gm. of CO_2, whilst an equal amount of it dried and dissolved up again to the same volume, gave 1·11 gm. of CO_2. The following data show how permanent is the stability of dried zymase :—

3 gm. Dried Juice (fresh) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave . . .	1·91 gm. CO_2.
3 gm. Dried Juice (kept 1 month) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	2·00 ,,
3 gm. Dried Juice (kept 2 months) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	2·07 ,,
3 gm. Dried Juice (kept 5 months) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	2·19 ,,
3 gm. Dried Juice (kept 7 months) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	1·76 ,,
3 gm. Dried Juice (kept 9½ months) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	2·03 ,,
3 gm. Dried Juice (kept 12 months) + 18 c.c. H_2O + 8 gm. Cane-Sugar + Toluol, in 168 hours at 17°, gave .	1·87 ,,

The thoroughly dried juice could be heated to 85° for eight hours without any substantial loss of activity, and even when kept for six hours at 97° it did not lose its activity entirely.

Zymase can be dried in quite another manner, viz., by precipitation with absolute alcohol, or a mixture of alcohol and ether. The precipitate must be collected on a filter, and the retained alcohol quickly removed by washing with ether. It is then dried *in vacuuo* over sulphuric acid, and the dried powder so obtained, if rubbed up in a mortar with cane sugar and the volume of water necessary to bring it up to the original volume

of juice, is found to possess nearly its initial activity. In two experiments, for instance, it yielded ·53 and 1·28 gm. of CO_2 respectively, as against ·54 and 1·33 gm. of CO_2 in the corresponding experiments with fresh juice.

The effects of antiseptics upon the activity of zymase and of living yeast cells have been studied by several investigators, and the results of such study are instructive, as they demonstrate very clearly that unorganised ferments and living organisms differ from one another only in degree in their reaction to antiseptics. Both are affected to some extent, and zymase, being more unstable in most respects than other enzymes, is as a rule affected more than they are by antiseptics; but even living organisms, though much more sensitive than zymase, can retain vitality in the presence of low concentrations of antiseptics.[1]

Buchner and Rapp[2] divide antiseptics into two classes, viz., those which enter into chemical combination with the proteins of the yeast juice, and those which do not enter into combination. In the first class fall corrosive sublimate and probably ammonium fluoride, both of which produce a precipitate when added to yeast juice. Potassium metarsenite may perhaps be included as well, for it produces a precipitate when in tolerable concentration. These bodies exert a harmful influence in proportion to the concentration they bear to the total amount of protein present in the juice, or of protoplasm in the living organism. In the second class of antiseptics fall concentrated glycerin and sugar, toluol, thymol, and chloroform. These are substances which do not combine with proteins, and which are supposed to exert a harmful influence in proportion to their absolute concentration, apart from their amount and the amount of

1 gm. Yeast + 50 c.c. 8 per cent. Cane-Sugar + ·5 gm. $CHCl_3$	= ·152 gm. CO_2 in 22 hours.
10 gm. Yeast + 50 c.c. 8 per cent. Cane-Sugar + ·5 gm. $CHCl_3$	= ·515 gm. CO_2 in 1 hour.
1 gm. Yeast + 50 c.c. 10 per cent. Cane-Sugar + ·5 gm. Toluol	= ·017 gm. CO_2 in 24 hours.
10 gm. Yeast + 50 c.c. 10 per cent. Cane-Sugar + ·5 gm. Toluol	= ·709 gm. CO_2 in 24 hours.

[1] *Cf.* Lecture VIII., p. 215. [2] Buchner and Rapp, *Ber.*, 32, p. 127, 1899.

protein present in the juice or yeast cells. However, Abeles[1] has obtained results which seem to disprove this hypothesis, for the data adduced show that with the larger quantity of yeast CO_2 was evolved at a relatively much faster rate than with the smaller quantity, when this was acting in the presence of the same amount of chloroform or toluol. No explanation of this result was arrived at, but probably the larger quantity of yeast did not become saturated with the antiseptic so quickly as the smaller quantity.

Of the first class of antiseptics, ammonium fluoride proved itself especially harmful to the activity of zymase, as the addition of ·55 per cent. of it to the juice paralysed the enzyme completely. Bokorny[2] found that living yeast cells were much more sensitive still, as ·1 to ·5 per cent. of it killed them. The action of arsenious acid, dissolved in K_2CO_3 and so partly converted into $KAsO_2$, was somewhat irregular, as is indicated by the following data:—

	CO_2 evolved in grammes.						Mean.
							gm.
20 c.c. Juice + 8 gm. Sugar alone	·90	·57	·48	·42	·42	·42	·54
20 c.c. Juice + 8 gm. Sugar alone + 2 per cent. As_2O_3	·43	·49	·63	·69	·37	·38	·50

These were obtained with different samples of juice, which were allowed to act upon cane-sugar for forty hours at about 17°. Upon diluted juice potassium metarsenite had a much more harmful influence, but this influence could be to a large extent neutralised by the addition of protein. The proteins of yeast juice, previously inactivated by heating for ten minutes to 55°, gave the best result of all, as the following data show:—

In 64 hours at 16°, 20 c.c. Juice + 16 gm. Sugar + 2 per cent. As_2O_3 + 20 c.c. Water gave . . . . ·14 gm. CO_2.
In 64 hours at 16°, 20 c.c. Juice + 16 gm. Sugar + 2 per cent. As_2O_3 + 20 c.c. Egg White gave . . . ·18 „
In 64 hours at 16°, 20 c.c. Juice + 16 gm. Sugar + 2 per cent. As_2O_3 + 20 c.c. Blood Serum gave . . . ·55 „
In 64 hours at 16°, 20 c.c. Juice + 16 gm. Sugar + 2 per cent. As_2O_3 + 20 c.c. Inactivated Yeast Juice gave . . ·86 „

[1] Abeles, *Ber.*, 31, p. 2261, 1898. [2] Bokorny, *Pflüger's Arch.*, 111, p. 371.

Analogous results to these have been obtained by Abeles with living yeast cells. A mixture of 1 gm. of yeast with 50 c.c. of nutritive solution containing 8 per cent. of cane-sugar and 1 gm. of sodium metarsenite evolved only ·002 gm. of CO_2 in three hours at 30°. On the other hand, 20 gm. of yeast mixed with 20 c.c. of water, 10 gm. of sugar and 1 gm. of sodium metarsenite evolved no less than 3·62 gm. of CO_2 in twenty-six hours. In this instance probably the protoplasm of a portion of the yeast cells combined with the arsenite, and the remaining cells formed CO_2, whilst in the case of yeast juice the proteins, both those already present and those added artificially, similarly combined with much of the arsenite, and protected the zymase.

Of the second class of antiseptics chloroform and toluol were found to exert comparatively little influence, but thymol was much more harmful, as is shown by these two sets of fairly concordant data:

	CO_2 evolved.	
	gm.	gm.
20 c.c. Juice + 8 gm. Sugar + no Antiseptic, in 92 hours gave .	1·24	1·84
„ „ + ·2 c.c. Toluol „ „ .	1·00	1·87
„ „ + 1 c.c. „ „ „ .	1·04	1·69
„ „ + ·2 gm. Thymol „ „ .	·74	1·18
„ „ + 1 gm. „ „ „ .	·62	·71

In remarkable contrast to the effect of toluol upon zymase, is its action upon living yeast cells. Buchner found that 1 gm. of living yeast cells, added to 15 c.c. of water + 5 c.c. of beer wort + 4 gm. of cane-sugar + ·2 c.c. of toluol, evolved only ·03 to ·05 gm. of CO_2 when kept for ninety-six hours at 22°. In control experiments without toluol, the same quantity of yeast gave 2·10 to 2·13 gm. CO_2: *i.e.*, forty to seventy times as much. It is to be remembered, however, that in the absence of toluol the yeast cells would be continually reproducing themselves and generating fresh zymase, whilst in the presence of toluol there would be no mechanism for increasing the store of zymase originally present. This store would be somewhat greater than that obtained in the juice of the ground-up cells, for doubtless a good deal of the enzyme is retained in the press cake. Arguing from the known chemical composition of yeast and from the fact

that a kilogram of ground yeast cells yields 500 c.c. of juice, we may assume that the total amount of juice actually present in them is about 700 c.c. One gramme of yeast would therefore contain ·7 c.c. of juice. Now Buchner found that 100 c.c. of juice gave off from 3·5 to 12·2 gm. of CO_2 under the most favourable circumstances, so the juice in 1 gm. of yeast would give off ·024 to ·085 gm of CO_2, or about the amount actually obtained from 1 gm. of yeast kept with sugar, beer wort, and toluol. Hence the only legitimate deduction that can be made from these comparative observations is that toluol stops all reproductive activity and regeneration of zymase in living yeast cells. They cannot be taken to show that toluol paralyses the formation of CO_2 by the intracellular zymase of an intact but dead yeast cell any more than it paralyses the action of the zymase in press juice.

A number of interesting observations have been made by Albert[1] and by Buchner which seem to show that the powers of actual reproduction and of formation of intracellular zymase by yeast cells are independent of one another, and that the one power may be destroyed without the other. Albert found that if yeast were put in a mixture of absolute alcohol and ether (250 gm. yeast, 3 l. alcohol and 1 l. ether) for five minutes, were then washed with ether and dried in air at 20° to 45°, the yeast cells were rendered sterile. But if suspended in cane-sugar solution in presence of toluol they evolved ten or even twenty times more CO_2 than is evolved by the juice pressed from an equal weight of ground-up yeast cells. For instance, 2 gm. of this sterile yeast +4 gm. sugar +10 c.c. water +2 gm. toluol gave ·92 to 1·05 gm. CO_2 in ninety-six hours at 22°. Again, Albert, Buchner, and Rapp[2] found that yeast could be rendered sterile by keeping it in acetone for ten minutes, and then washing with ether and drying. Another method consisted in heating the carefully dried yeast for six hours to 100°. In each case the yeast, when placed in sterile beer wort, failed to show any signs of reproduction, but still retained this considerable fermentative power. The retention of this power is difficult to reconcile with the above-mentioned fact that living yeast cells, when kept under similar conditions in presence

[1] Albert, *Ber.*, 33, p. 3775, 1900.

[2] Albert, Buchner, and Rapp, *Ber.*, 35, p. 2376, 1902.

of toluol, yielded no more CO_2 than the press juice which could be extracted from them; for this shows that the toluol paralyses the zymase-forming power of the cells, as well as the reproductive power. Presumably these two functions are so intimately bound up together in the living cell that if the one is paralysed the other is likewise, but in the sterilised cell the destruction of the one leaves the other independent. Of course it is possible though not probable that the fermentative power of sterilised yeast is due entirely to zymase present from the outset in the sterilised cells, and that there is no new formation of zymase whatever. If this is the case, then the much smaller activity of press juice implies that the major part of its zymase is destroyed during the violent mechanical methods of extraction.

Zymase, in addition to its extreme instability, differs from other enzymes in that its activity seems to be absolutely dependent on the presence of phosphates. Wroblewski[1] noticed that disodium phosphate exerted a favourable influence on zymase activity, the addition of 1·25 per cent. of it giving the best result. Buchner found that any concentration up to 4 per cent. of it acted equally well, and that its addition increased the output of CO_2 by over 30 per cent. Harden and Young[2] obtained still more striking effects by the addition of the phosphates of the juice itself. They found that if boiled and filtered juice were added to fresh juice, together with glucose and toluol, its CO_2 output might be doubled or trebled. On the other hand, if the phosphates already present in the juice were removed, it entirely lost its fermenting power. A fairly complete separation of the colloidal and crystalloidal constituents of the juice was effected by filtration through a Martin gelatin filter. A saline filtrate containing no enzyme was obtained thereby, whilst a brown viscid mass was left on the filter. This residue was dissolved in water and made up to the volume of the juice filtered, but it yielded little or no CO_2 when kept with glucose. On addition of the saline filtrate, however, it recovered a good deal of its initial activity. Buchner and Antoni[3] repeated this experiment

[1] Wroblewski, *Journ. f. prakt. Chem.* (2), 64, p. 11, 1901.
[2] Harden and Young, *Proc. Roy. Soc.*, B. 77, p. 405, 1906.
[3] Buchner and Antoni, *Zeit. f. physiol. Chem.*, 46, p. 136, 1905.

in another form. They separated the salts from the juice by dialysis, and found that the residue in the dialysis tube had little or no fermenting power until some of the dialysed salts were added. Thus:—

20 c.c. Dialysed Juice	+ 10 c.c. Water + 8 gm. Cane-Sugar gave	·02 gm.	CO_2.
„ „	+ 10 c.c. Evaporated Dialysate gave .	·48	„
„ „	+ 20 c.c. Boiled Juice gave . .	·59	„

The increased evolution of CO_2 produced by the addition of phosphates to yeast juice *plus* sugar was found by Harden and Young to be within certain limits strictly proportional to the phosphates added. Each atom of phosphorus added as phosphate (a solution of Na_2HPO_4 and NaH_2PO_4 saturated with CO_2) caused the evolution of a molecule of CO_2. For instance, in four experiments the amounts of CO_2 calculated on this basis were ·055, ·086, ·112, and ·197 gm. respectively, whilst the amounts actually observed were ·054, ·090, ·106, and ·196 gm. respectively. The mode of action of the phosphates is unknown. It might be thought that the CO_2 was formed in the usual way by the zymase, but that it remained in loose combination with certain constituents of the juice, presumably the protein constituents, and was only liberated therefrom by an equivalent amount of phosphate taking its place. However, recent investigation indicates that the reaction is not so simple as this, for Harden and Young[1] found that the addition of soluble inorganic phosphates to a solution of the inactive yeast juice residue was quite unable to provoke fermentation. Apparently the fermentation depends on the presence of some crystalloidal thermostable "co-enzyme" in the yeast juice as well as on the phosphates. Again, Buchner and Klappe[2] found that if fresh yeast juice were kept for three or four days with sugar and toluol, until its CO_2 output had ceased, it was still able to give a very considerable further output of CO_2 if boiled juice were added gradually. In one experiment 20 c.c. of the fresh juice gave out ·73 gm. of CO_2 altogether. Then volumes of 20 c.c. of boiled juice, together with sugar and toluol, were added on seven successive occasions

[1] Harden and Young, *Proc. Roy. Soc.*, B. 78, p. 369, 1906; 80, p. 263, 1908.

[2] Buchner and Klappe, *Biochem. Zeit.*, 8, p. 520, 1908.

at two- to five-day intervals, and they provoked a further discharge of ·32, ·17, ·42, ·29, ·19, ·07, and ·05 gm. of CO_2 respectively, or 1·5 gm. of CO_2 in all. It follows, therefore, that some of the zymase must have remained active for as long as twenty-seven days. The addition of boiled juice to yeast juice which had been kept standing without any sugar for three days failed to provoke any CO_2 output, and also this inactive juice, when boiled, was found to have lost its activating power upon juice kept with sugar. As the result of some not very convincing experiments, Buchner and Klappe conclude that the co-enzyme in boiled juice may be an organic ester of phosphoric acid, which in kept (sugarless) juice is split up and rendered inactive by the action of a lipase.

The molecular changes involved in the conversion of sugar into alcohol and CO_2 are not known, but recent research seems to point with some probability to there being at least two stages in the process. Buchner and Meisenheimer[1] suggest that each molecule of glucose is first converted into two molecules of lactic acid, and that this lactic acid is subsequently broken down into alcohol and CO_2. The experimental support for this hypothesis is at present somewhat slender. It depends chiefly on the fact, first noted by Meisenheimer,[2] that yeast juice forms quite appreciable quantities of lactic acid. Buchner and Meisenheimer estimated the percentage of the acid in a number of samples of juice, and they found that whilst fresh juice contained ·01 to ·14 per cent. of the acid, juice which had undergone autolysis for four to six days contained ·09 to ·40 per cent. of acid. The addition of sugar to the juice made very little difference, so probably the lactic acid was formed at the expense of intracellular glycogen. On repeating these observations during the summer months of two successive years, Buchner and Meisenheimer found that the small quantities of lactic acid originally present in the juice disappeared on autolysis, and that if some lactic acid were added to the juice it was likewise destroyed. It seemed probable, therefore, that the juice contained variable proportions of two distinct enzymes, a "zymase,"

[1] Buchner and Meisenheimer, *Ber.*, 37, p. 417, 1904; 38, p. 620, 1905.

[2] Meisenheimer, *Zeit. f. physiol. Chem.*, 37, p. 526, 1903.

which has the power of converting sugar into lactic acid according to the equation

$$C_6H_{12}O_6 = 2C_3H_6O_3,$$

and a "lactacidase" enzyme, which splits up the lactic acid into $C_2H_6O + CO_2$. It is supposed that in the winter months the lactacidase was lacking in amount, whilst in the summer months it was in excess. In that living yeast cells form scarcely a trace of lactic acid, it would seem that in their case there is always plenty of lactacidase available.

Attempts have been made to isolate a lactic acid-forming enzyme from various bacteria. Herzog[1] found that if lactic acid bacteria were killed by immersion in acetone, they were still able to split up milk sugar and form a body which he identified, not altogether satisfactorily, as lactic acid. Buchner and Meisenheimer[2] used a preparation of *Bacillus Delbrücki* which had been placed for ten to fifteen minutes in acetone and washed with ether. They found that it readily formed lactic acid when placed in cane-sugar or maltose in presence of toluol. Ten grammes of the preparation yielded ·75 to 1·26 gm. of lactic acid. A control experiment with bacteria previously heated to 91° gave no lactic acid at all. Somewhat unexpectedly the lactic acid formed was found to be the optically inactive variety, whilst the living bacillus produces the lævo-rotatory acid. Juice expressed from the ground-up bacteria showed no lactic acid-forming power, though the press cake, even after treatment with acetone, still retained its activity. This seems to show that the enzyme is insoluble, or more probably, as Buchner and Meisenheimer think, that the active constituents of the bacterial cells are not pressed out in the juice. It seems very unlikely, however, that *none* of the enzyme should be driven out by the forcible mechanical means adopted, so one is perforce driven to question the validity of the whole evidence. It is true that these "Acetondauerpräparate" of yeast cells and of bacteria, if placed in a sterile nutrient medium, show no signs of reproductive power, but must one on that account look upon them as dead? Reproduction in all organisms calls for the

[1] Herzog, *Zeit. f. physiol. Chem.*, 37, p. 381, 1903.
[2] Buchner and Meisenheimer, *Liebig's Ann.*, 349, p. 125.

highest degree of vitality, and if this vitality be reduced to a low ebb by treatment with acetone and ether, it may be insufficient to bring about growth and reproduction, though still adequate for the normal metabolic processes of the cell. On the other hand, we must remember that these acetone preparations act perfectly well in the presence of toluol. "Dauerhefe," for instance, evolves ten times as much CO_2 as living yeast kept under similar conditions. Again, it is possible that the lactic acid-forming enzyme is so unstable that it is destroyed by the act of forcible rupture from its protoplasmic basis in the cell.

The action of inorganic catalysts such as caustic alkalis upon sugar seems to throw light upon the processes of decomposition by enzymes. As long ago as 1871 Hoppe-Seyler[1] and Schützenberger showed that caustic alkalis split up sugar with the formation of a considerable amount of lactic acid. Nencki and Sieber[2] found that if a 10 per cent. glucose solution were incubated with 20 per cent. of KOH for twenty-four hours, it was almost completely decomposed, and as much as 50 per cent. of it was converted into lactic acid. More dilute alkali effected a similar decomposition but at a slower rate, and even ·3 per cent. KOH split it up in ten days at 37°. Duclaux[3] found that if glucose were kept in sunlight in presence of weak alkalis such as baryta or lime water, some 50 per cent. of it was converted into lactic acid, but if the weak alkali were replaced by a strong one such as KOH, then alcohol and CO_2 were formed. He suggested that lactic acid was formed in every case, but that only a strong alkali was able to break it down further. Duclaux likewise found that if an aqueous solution of calcium lactate were kept in sunlight in presence of air, it formed alcohol, calcium carbonate, and calcium acetate. Hanriot[4] stated that if calcium lactate were heated with excess of calcium hydrate, considerable quantities of ethyl alcohol and acetone were produced. Buchner and Meisenheimer[5] confirm this

[1] Hoppe-Seyler, *Ber.*, 4, p. 396, 1871.

[2] Nencki and Sieber, *Journ. f. prakt. Chem.*, 24, p. 502, 1881.

[3] Duclaux, *Ann. de l'Inst. Nat. Agronomique*, 10, 1886. *Ann. de l'Inst. Pasteur*, 7, p. 751, 1893; 10, p. 168, 1896.

[4] Hanriot, *Bull. Soc. Chim.*, 43, p. 417, 1885; 45, p. 80, 1886.

[5] Buchner and Meisenheimer, *Ber.*, 38, p. 620, 1905.

statement, but they find that a good deal of isopropyl alcohol is formed as well. They also confirm the experiments of Duclaux, and find that even at room temperature and in darkness caustic potash slowly converts glucose into lactic acid and other products.

Of the intermediate stages between glucose and lactic acid we know nothing for certain, but probably the glucose first breaks up into two molecules of glyceric aldehyde, $CH_2OH-CHOH-CHO$. Nef[1] has shown that in the conversion of glucose into lactic acid by the action of caustic alkali, pyruvic aldehyde, $CH_3-CO-CHO$, is formed as an intermediate product, and Buchner and Meisenheimer suppose that this substance likewise represents a stage in the conversion of glyceric aldehyde into lactic acid by zymase. Such an assumption makes the molecular changes more complex than if the glyceric aldehyde be supposed to pass directly into lactic acid, so it should not be adopted unless supported by stronger evidence than is at present available. In the conversion of lactic acid into alcohol and CO_2, it is probable that acetic aldehyde and formic acid are first produced:

$$CH_3-CH.OH-COOH = CH_3-CHO+H.COOH.$$

The formic acid then breaks up into CO_2 and hydrogen, and this hydrogen reduces the aldehyde to alcohol. Thus lactic acid splits up into aldehyde and formic acid if heated with dilute sulphuric acid to 130°, or if electrolysed.[2]

In the autolysis of yeast juice acetic acid is formed as well as lactic acid. Buchner and Meisenheimer found ·03 to ·28 per cent. of this acid in the juice after four to six days autolysis. They attribute its formation to an alcohol-oxidase enzyme.[3] It seems probable that yeast juice contains other enzymes in addition to those recorded. Pasteur found that in the fermentation of sugar by living yeast, there were always certain amounts of glycerin and succinic acid formed. These amounts varied under different conditions, and the slower the fermentation the greater they were, but as a rule the glycerin formed 2·5 to 3·6 per cent. on the weight of sugar fermented, and the succinic acid ·5 to ·7 per cent. Buchner found that these two substances are likewise produced in the fermentation of yeast juice, though in

[1] Nef, *Annalen*, 335, p. 247, 1904.

[2] Erlenmeyer, *Zeit. f. Chem.*, 1868, p. 343. [3] See Lecture V., p. 126.

smaller proportion. Thus 1250 c.c. of juice gave ·5 gm. of glycerin and ·3 gm. of succinic acid. Buchner and Meisenheimer[1] subsequently found that no succinic acid is formed as a rule, but that the glycerin amounted to from 5·4 to 16·5 per cent. on the sugar fermented.

The isolation of a glycolytic enzyme from yeast suggested that the glycolytic powers possessed by all living tissues might depend, wholly or in part, on a similar enzyme, whilst the alcohol formed by the action of this zymase was supposed to be split up by another enzyme into CO_2 and water. In support of this hypothesis, Blumenthal[2] found that the juice expressed from the pancreas has a strong glycolytic action upon glucose. Carbon dioxide was formed thereby, but Blumenthal could not demonstrate the formation of alcohol. Umber[3] could not confirm the formation of CO_2, but Herzog[4] obtained doubtful evidence of it. In 1903 Stoklasa,[5] working in conjunction with Jelinek, Czerny, and Vitek, stated that he had been able to isolate an active zymase, not only from the roots and seeds of plants, but also from several different animal tissues. He demonstrated the existence of this animal zymase by several methods. The simplest method consisted in dipping pieces of the tissue (heart, liver, lungs, and muscle) in ·5 per cent. corrosive sublimate solution for fifteen to thirty minutes to render them aseptic, and then placing them in a sterile 5 per cent. glucose solution at a temperature of 37°. The vessel containing the glucose was filled with hydrogen, so the tissue enzyme was acting under anaërobic conditions. Fermentation was well established within twenty-four hours, and continued for some days. A dog's heart weighing 21 gm. caused the evolution of ·3 to ·5 gm. of CO_2 per day for the first three days, and 1·97 gm. in all during ten days. The alcohol formed at the same time amounted to 2·09 gm., so a normal alcoholic fermentation seemed to have occurred. In that gelatin and bouillon cultures failed

[1] Buchner and Meisenheimer, *Ber.*, 39, p. 3204, 1906.

[2] Blumenthal, *Zeit. f. diät. u. phys. Therap.*, vol. ii.

[3] Umber, *Zeit. f. klin. Med.*, 39, p. 13.

[4] Herzog, *Hofmeister's Beitr.*, 2, p. 102, 1902.

[5] Stoklasa and Czerny, *Centralb. f. physiol.*, 16, p. 652, 1903; Stoklasa, Jelinek, and Czerny, *ibid.*, 16, p. 712; Stoklasa, Jelinek, and Vitek, *Hofmeister's Beitr.*, 3, p. 460, 1903.

to show the presence of bacteria or hyphomycetes, Stoklasa concluded that the fermentation of the glucose was effected by an intracellular zymase.

To isolate the enzyme, Stoklasa adopted the method used by Buchner for yeast zymase. A mixture of alcohol and ether was added to the press juice obtained from the minced tissue, and the precipitate was quickly washed with ether, dried, and powdered. In one experiment 9·64 gm. of the powder obtained from muscle juice was placed in 15 per cent. glucose solution at 37°, and in eighteen hours it formed ·73 gm. of CO_2 and ·78 gm. of alcohol. In another experiment 10 gm. of ox lung powder gave 1·19 gm. of CO_2 during the first twelve hours of fermentation, and 3·09 gm. of CO_2, together with 3·20 gm. of alcohol, in fifty hours. Stoklasa subsequently found that considerable quantities of lactic acid were formed, as well as alcohol and CO_2. For instance, 10 gm. of muscle powder, placed in 50 c.c. of 15 per cent. glucose at 37°, formed 2·7 gm. of CO_2, 2·8 gm. of alcohol, and 1·7 gm. of lactic acid. Liver and lung powder gave a similar result, and even the alcohol-ether precipitate of blood induced a moderate amount of alcoholic fermentation in glucose, but it formed only a small amount of lactic acid. Most active of all in producing alcoholic fermentation was pancreas powder. Simácek[1] found that ·164 gm. of this powder, placed in 15 per cent. glucose solution at 37°, gave ·42 gm. of CO_2 and 1·12 gm. of alcohol in four days. It could likewise ferment cane-sugar, maltose, and lactose. For instance, 5 gm. of pancreas powder, placed in 50 c.c. of 30 per cent. lactose at 36° for seventy-two hours, gave ·846 gm. CO_2, ·122 gm. alcohol, and had an acidity corresponding to 1·08 gm. of lactic acid.

All of these experiments were made under anaërobic conditions, and, according to Stoklasa and his colleagues, in the entire absence of bacterial infection. Could we accept them unreservedly, they would be of great service in helping us to understand, not only the processes of glycolysis in the body, but also the processes of tissue respiration. Unfortunately we are unable to put our faith in them at present, as most other investigators have been unable to confirm them. Mazé[2] found

[1] Simácek, *Centralb. f. Physiol.*, 17, pp. 3 and 209, 1903.
[2] Mazé, *Ann. de l'Inst. Pasteur*, 18, p. 378, 1904.

that the alcohol-ether precipitate from the expressed juice of ox lung, and that from pounded peas, caused a vigorous fermentation in glucose solution with the formation of CO_2, alcohol, and lactic acid, but bacteria were always present. Also the first portions of the gas evolved consisted largely of hydrogen. Battelli[1] found that if only sufficient antiseptic were present, not a trace of fermentation resulted. Portier[2] found that the precipitate from the expressed juice of various organs of the dog, pig, and horse, when placed in glucose solution containing 1 per cent. NaF, did not produce any glycolysis in two days at 36°. Also he points out that the fermentation of cane-sugar and lactose by pancreas juice, which Simácek observed, implies the presence of invertase and lactase ferments in the pancreas. As was mentioned in the previous lecture, it is practically certain that no lactase whatever exists in this organ, and it is very doubtful if any invertase does either.

Hence until more satisfactory evidence is brought forward, we are not justified in assuming that animal tissues contain enzymes producing alcoholic fermentation. The fact that Stoklasa and his co-workers could not detect bacteria by means of their cultures does not necessarily prove that they did not exist. As far as one can gather from their papers, the cultures were as a rule made under aërobic conditions, though the fermentations were anaërobic. Hence the cultural conditions may not have been favourable for growth of the bacteria which induced the alcoholic fermentation.

On the other hand, there is a good deal of evidence that glycolytic enzymes of some sort exist in animal tissues. The evidence of Blumenthal has already been quoted. Arguing from the well-known discovery of v. Mering and Minkowski that extirpation of the pancreas causes diabetes, Cohnheim[3] endeavoured to prove that the glycolytic power of the muscles is dependent on an internal secretion of the pancreas. He found that if glucose were incubated with muscle press juice or with pancreas press juice, little or none of it disappeared. If, on the other hand, fresh muscle and pancreas were minced together, the

[1] Battelli, *Comptes Rendus*, 137, p. 1079, 1903.
[2] Portier, *Ann. de l'Inst. Pasteur*, 18, p. 633, 1904.
[3] Cohnheim, *Zeit. f. physiol. Chem.*, 39, p. 336, 1903.

mixed juice squeezed out from the two tissues possessed distinct glycolytic power. For instance, samples of the mixed juice when incubated for a considerable time with about 2 per cent. of glucose induced a destruction of ·35 to ·84 per cent. of the sugar, whilst the juice of muscle alone, kept with glucose under similar conditions, destroyed 0 to ·026 per cent., and the juice of pancreas alone destroyed no sugar at all. Toluol was added to prevent bacterial action, whilst the acidity of the juice was neutralised by sodium bicarbonate. Subsequently Cohnheim[1] showed that the activating power of the pancreas is not dependent on an enzyme, in that aqueous or alcoholic extracts of boiled pancreas were equally efficient. In order to obtain the best results, only a moderate amount of pancreatic extract or pancreas juice must be added to the muscle juice, as an excess of it exerts an inhibitory influence. It was found, for instance, that muscle juice alone destroyed ·034 gm. of glucose. On addition of 10 c.c. of aqueous extract of pancreas to 40 c.c. of juice it destroyed ·115 gm.; on addition of 20 c.c., it destroyed ·174 gm.; of 28 c.c., ·093 gm.; and of 50 c.c., none whatever. The activating body is present in the blood as well as in the pancreas, so that if the muscles are not thoroughly washed free of blood, their juice has considerable glycolytic power.

These very interesting experiments have been repeated by Claus and Embden,[2] but they found little or no glycolysis unless bacteria were present. Cohnheim attributes their non-success to the fact that they used sodium chloride in preparing their muscle juice, and added too much pancreatic extract. De Meyer[3] concluded that a glycolytic enzyme is present in the blood and lymph alone, and does not occur in the tissue cells. It is formed, he considers, by the interaction of an activating body (an amboceptor) from the islands of Langerhans of the pancreas with a zymogen secreted by the leucocytes of the blood. De Witt[4] ligatured the duct of a portion of the cat's pancreas, and 49 to 197 days later, when all but the islands of

[1] Cohnheim, *Zeit. f. physiol. Chem.*, 42, p. 401, 1904; 43, p. 547, 1905; 47, p. 253, 1906.

[2] Claus and Embden, *Hofmeister's Beitr.*, 6, pp. 214 and 343, 1906.

[3] De Meyer, *Ann. Soc. Roy. de Sci. à Bruxelle*, 1906; Abstract in *Centralb. f. Physiol.*, 20, p. 348.

[4] De Witt, *Journ. of Exp. Med.*, 8, p. 123, 1906.

Langerhans had undergone atrophy, made extracts of it and of the healthy portions of the gland. These extracts, sometimes boiled, sometimes not, were mixed with muscle extract and ·7 to 4 per cent. of glucose, and in twenty-four hours ·1 to ·9 per cent. of this sugar disappeared. In that the extracts of atrophied gland conferred just as much glycolytic power on the muscle extract as those of healthy gland, De Witt concluded that the activating body is present in the Langerhans islands alone. The experimental evidence is not sufficiently complete, however, for one to accept it unreservedly.

A careful repetition and full confirmation of Cohnheim's work has been made by Hall.[1] Press juice of muscle and of pancreas, and alcoholic extract of boiled pancreas were employed, and were allowed to act singly or in combination upon 2 to 4 per cent. glucose solution for fifteen to seventy-two hours at 37° in presence of toluol. Reckoning the total sugar present as 100, Hall found that on an average the pancreas juice alone destroyed ·3 per cent. of it, muscle juice alone 1·6 per cent., pancreas *plus* muscle juice 4·4 per cent., and alcoholic extract of pancreas *plus* muscle juice no less than 18·3 per cent. These results strongly support Cohnheim's, for the negative results always obtained with pancreas juice alone, the consistently low results obtained with muscle juice alone, and the consistently high ones with pancreatic extract and muscle juice, render the chance of bacterial infection very improbable. Also in a number of cases both aërobic and anaërobic cultures were made, and all but one proved sterile. The glycolytic power of extract of pancreas and muscle juice upon fructose, lactose, and arabinose was tested, but with negative results.

Several observers have found that glycolytic power is by no means confined to the muscles and pancreas. Hirsch[2] stated that when the liver underwent autolysis in the presence of antiseptics, some of its carbohydrate disappeared, or if sugar were added, this diminished likewise. The glycolysis was greatly increased if minced pancreas were added, though the pancreas alone had no glycolytic power. Arnheim and

[1] Hall, *Amer. Journ. Physiol.*, 18, p. 283, 1907.

[2] Hirsch, *Hofmeister's Beitr.*, 4, p. 535, 1904.

Rosenbaum[1] made most of their observations with dried powders prepared from the tissue juices by precipitation with acetone, and subsequent washing with ether. A gramme of powder was kept with 20 c.c. of 4·6 per cent. glucose solution for twenty-four hours at 37° in presence of chloroform, and the loss of sugar during this period estimated by polarimeter. The mean percentages of sugar destroyed were the following :—

Muscle Powder alone		destroyed	10	per cent.
Liver	,,	,,	27	,,
Pancreas	,,	,,	36	,,
Pancreas + Muscle		,,	65	,,
Pancreas + Liver		,,	57	,,
Liver + Muscle		,,	16	,,

As in Cohnheim's observations, the pancreas powder greatly increased the glycolytic power of muscle. It increased that of liver as well, but contrary to Cohnheim and to Hall, the pancreas itself had a considerable glycolytic power.

In other observations Arnheim and Rosenbaum determined the weight of CO_2 evolved in twenty-four hours by an incubated mixture of 1 or 2 gm. of powder with 30 c.c. of 10 per cent. glucose solution. The data show that there was a small evolu-

Pancreas Powder alone + Glucose Solution				lost	·04, ·06, ·14, ·16, ·10 gm. CO_2
Muscle	,,	,,	,,	,,	·14 gm.
Pancreas + Muscle	,,	,,	,,	,,	·66, ·24 gm.
Pancreas + Liver	,,	,,	,,	,,	·44, ·62, 1·01 gm.

tion of CO_2 from the pancreas or muscle alone, but quite a considerable one from the pancreas *plus* muscle and pancreas *plus* liver. These experiments are not directly comparable with those of the previous series, but they bear a similar relationship to one another, and show that a large proportion of the decomposed glucose was converted into CO_2.

In almost all cases the absence of bacterial infection was proved by cultures on gelatin and agar, made under both aërobic and anaërobic conditions, and antiseptics such as toluol or chloroform were always added: hence one may at least provisionally accept the results. It is by no means unlikely that there was a similar evolution of CO_2 in Cohnheim's and

[1] Arnheim and Rosenbaum, *Zeit. f. physiol. Chem*, 40, p. 220, 1903.

Hall's experiments, but no direct observations on the point were made.

The glycolytic power of the press juice of pancreas, liver, and muscle, and also of their alcohol-ether precipitates, has been observed by Feinschmidt.[1] Even in the presence of ·9 per cent. NaF a considerable glycolysis occurred. Large quantities of CO_2 were evolved, and a considerable acidity developed in the digestion liquid. Alcohol was shown to be present by qualitative tests, but the amount was so small that Feinschmidt considers that the fermentation should not be spoken of as an alcoholic one.

Evidence of quite another character in favour of an alcohol-producing enzyme in blood and animal tissues has been brought forward by Ford.[2] As long ago as 1858 Ford stated that traces of alcohol are normally present in blood and the tissues. To demonstrate its existence, the blood was obtained fresh from the slaughter-house, and immediately subjected to distillation. The organs were similarly treated after being chopped up. The first distillate was purified and concentrated by successive distillations, often twelve or more in number, until a final distillate of 1 to 3 gm. was obtained. The alcohol in this was estimated quantitatively by a specific gravity determination, and qualitatively by the chromic acid test, and ignition of the vapour of the boiling alcohol. Samples of blood varying from 6970 to 36,300 c.c. were analysed, and were found to contain ·0020 to ·0156 gm. of alcohol per kilogram. Probably a good deal of the alcohol originally present in the blood disappears as the result of post-mortem oxidation, for blood to which a strong solution sulphuretted hydrogen was added, so as to abolish this oxidation, gave nearly double the yield of alcohol. Fresh ox liver gave only ·0017 gm. of alcohol per kilogram, or no more than would be present in the blood of the organ. Pancreas and lung tissue likewise contained traces of alcohol.

The glycolytic power of the blood is well known. Claude Bernard found that the blood of a dog when fresh contained ·107 per cent. of sugar. After standing for thirty minutes at 15°, it had dropped to ·081 per cent.; after five hours, to ·044 per

[1] Feinschmidt, *Hofmeisters Beitr.*, 4, p. 511, 1904.

[2] Ford, *Journ. Physiol.*, 34, p. 431, 1906.

cent.; whilst after twenty-four hours it had entirely disappeared. Subsequent observers have confirmed Bernard's result, and they attribute the glycolysis to the action of an enzyme. According to Arthus[1] this enzyme arises from the leucocytes, on their post-mortem disintegration. It is not present in blood plasma.[2] It is generally assumed that this enzyme in an oxidase, and Seegen[3] states that its action is favoured by aëration of the blood. In any case it is possible that the sugar is in part converted into alcohol. Oppenheimer[4] endeavoured to obtain evidence of zymase in blood, and he found that if fresh blood were allowed to stand with sugar, and were distilled, small quantities of a substance were obtained which gave the iodoform test, and which was not acetone. The fresh blood also gave a feeble iodoform test, so it seems very probable that alcohol was originally present, and that more was formed in the kept blood.

The presence of alcohol in the distillate from brain, muscle, and liver of the rabbit and horse was demonstrated by Rajewsky[5] in 1875. The distillate readily gave the iodoform test, and in the presence of platinum black formed aldehyde, but no quantitative estimations were attempted. Again, Kobert[6] found that if the yolk of tortoise eggs, or sea-urchin's ova, were ground up into an emulsion with 1 per cent. sodium fluoride solution containing toluol, and the mixture were kept in an incubator with glucose for four or five days, and then distilled, the distillate contained appreciable quantities of alcohol. Kobert tested for it by the iodoform and chromic acid tests, but did not estimate it quantitatively. He also found that if *Ascaris* were ground up with kieselguhr, and the mixture incubated for sixteen hours with sodium fluoride and toluol solution, it gave an alcohol-containing distillate. A similar result was obtained with earthworms, and hence Kobert concludes that these organisms, and also the ova, contain zymase.

Though for the present we cannot definitely accept or reject the presence of an enzyme of alcoholic fermentation in animal

[1] Arthus, *Arch. de physiol.* (5), 3, p. 425, 1891; (5), 4, p. 337, 1892.
[2] Doyen and Morel, *C. R. Soc. Biol.*, 55, p. 215, 1903.
[3] Seegen, *Centralb. f. Physiol.*, 5, pp. 821 and 869, 1891.
[4] Oppenheimer, *Die Fermente*, 2nd ed., Leipsig, 1903, p. 320.
[5] Rajewsky, *Pflüger's Arch.*, 11, p. 122, 1875.
[6] Kobert, *Pflüger's Archiv*, 99, p. 116, 1903.

tissues, we can speak with greater confidence with regard to vegetable tissues. It was shown by Rollo[1] that the higher plants formed alcohol if they were were kept in absence of oxygen, and Pasteur[2] and many other investigators confirmed this observation. Hence it has been suggested that the anaërobic respiration of plants is no more or less than alcoholic fermentation. In support of this hypothesis Polzeniusz and Godlewski[3] showed that the anaërobic respiration of pea seeds corresponded to the equation:

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O$$

whilst Godlewski[4] observed the same thing for lupin seeds. Nabokich[5] repeated the experiments, and found that the ratio of CO_2 to alcohol did not always agree with that of alcoholic fermentation. Sometimes the alcohol amounted to only 50 per cent. of the theoretical.

Recently Palladin and Kostytschew[6] investigated and compared the anaërobic respiration of living and dead seeds and plants. The seeds were killed by placing them in a U-tube, and cooling them to a temperature of $-20°$ to $-3°$ for twenty-four hours. A current of hydrogen was then led through, and the CO_2 given off collected in baryta water. It appeared that though the anaërobic respiration of living lupin seeds, germinating or otherwise, corresponded fairly closely with alcoholic fermentation, that of the dead seeds did not. They continued to evolve CO_2 for a good many hours, but formed little or no alcohol. For instance, 100 gm. of living lupin seeds in twenty-four hours at 20° evolved ·160 gm. of CO_2 and ·145 gm. of alcohol, whilst 100 gm. of previously frozen seeds evolved ·083 gm. of CO_2, but no alcohol whatever. In contrast to lupin seeds, castor-oil and pea seeds, and germinating wheat, showed a considerable formation of alcohol whether they were dead or alive. Living castor-oil seeds formed CO_2 and alcohol in the

[1] Rollo, cited by Oppenheimer, *Die Fermente*, p. 319.

[2] Pasteur, *Comptes Rendus*, 75, p. 1056, 1872.

[3] Polzeniusz and Godlewski, *Bull. de l'Acad. d. Sci. d. Cracovie*, 1897, p. 267; 1901, p. 227.

[4] Godlewski, *ibid*, 1904, p. 115.

[5] Nabokich, *Ber. d. botan. Ges.*, 21, p. 467, 1903.

[6] Palladin and Kostytschew, *Zeit. f. physiol. Chem.*, 48, 214, 1906.

proportion of 100 to 60, and dead ones, in the proportion of 100 to 59, hence the respiration was not a typical alcoholic one in either case. Dead stalk tips and leaves of the vetch (*Vicia faba*) evolved CO_2 and alcohol in the proportion of 100 to 17; dead pea seeds, in the proportion of 100 to 75; and dead germinating wheat in the proportion of 100 to 93. It seems probable, therefore, that anaërobic respiration is in all cases similar to alcoholic fermentation, but that frequently other processes are at work which convert the alcohol first formed into some other products. In living organisms these products are CO_2 and water, provided that sufficient oxygen is available. Thus living pea seeds, in absence of oxygen, gradually accumulate alcohol, but in presence of oxygen, oxidise it completely. Frozen pea seeds accumulate a good deal of alcohol even in the presence of oxygen, so in their case the oxidising mechanism has been weakened or destroyed by the low temperature.

As zymase is almost certainly present in many seeds and plants, if not in all, one would expect that it could be isolated like yeast zymase. Stoklasa, Jelinek, and Vitek[1] find that the juice expressed from beetroot, if kept in hydrogen in presence 2 per cent. of potassium metarsenite, undergoes a very slow alcoholic fermentation. For instance, 500 c.c. of the juice, at a temperature of 22°, gave ·179 gm. of CO_2 and ·120 gm. of alcohol in six days. The precipitate thrown down by addition of alcohol *plus* ether to the juice was more active, as 6 gm. of it caused immediate fermentation in the 100 c.c. of 15 per cent. glucose solution to which it was added, and in forty-eight hours at a temperature of 30° formed ·64 gm. of CO_2 and ·94 gm. of alcohol. Subsequently Stoklasa, Ernest, and Chocenský[2] found that the alcohol-ether precipitate prepared from the juice of the root and leaves of the beet induced lactic acid fermentation. Thus 10 gm. of precipitate, kept in 15 per cent. glucose solution together with 1 or 2 per cent. of salicylic acid for forty-eight hours at 20°, gave ·08 to ·36 gm. of lactic acid, ·28 to ·86 gm. of CO_2, and ·21 to ·80 gm. of alcohol. The digestion liquids were tested and found to be sterile, but until confirmatory evidence

[1] Stoklasa, Jelinek, and Vitek, *Hofmeister's Beitr.*, 3, p. 460, 1903.

[2] Stoklasa, Ernest, and Chocenský, *Zeit. f. physiol. Chem.*, 50, p. 303, 1907.

has been obtained by other observers it is best not to accept this apparent isolation of plant zymase as proven. In the only other observations upon plant juices of which I find a record, an enzyme of alcoholic fermentation seemed to be lacking. Hahn[1] pounded up the spadices of the Cuckoopint (*Arum maculatum*) with sand and kieselguhr, and pressed out the juice. It contained a good deal of reducing sugar, and on standing a few days this entirely disappeared. For instance, 20 c.c. of juice obtained from the upper club-shaped part of the spadix contained ·19 gm. of sugar, but after six days at 25°, none whatever; whilst 20 c.c. of juice from the lower flower-carrying part of the spadix contained ·364 gm. of sugar, but after two days, only ·092 gm. This glycolysis was accompanied by some evolution of CO_2, but not enough to account for the sugar lost. A good deal of acid was formed, but never any alcohol.

It has been stated incidentally that in autolyses of both animal and plant tissues considerable acidity is developed. In most cases the nature of the acids formed, and their amount, have not been studied in detail. But Magnus-Levy[2] has done this for autolyses of dog and ox liver, and has obtained some very noteworthy results. In some experiments large pieces of liver (150 to 900 gm.) were kept under aseptic conditions for some hours or days at 38°, and in others the liver was minced and kept with twice its volume of saline, and an antiseptic. In the former series, the asepsis was verified by aërobic and anaërobic cultures. The aseptic liver became strongly acid in twenty-four hours, and formed a semi-fluid mass. It contained considerable quantities of non-volatile acids such as lactic and succinic acids, and of volatile acids such as formic, acetic and butyric. The data given in the table show the amounts of normal sodium hydrate solution required to neutralise the acids in 100 gm. of the gland substance. We see that after twenty-four hours' autolysis the ox liver needed 16·0 c.c. of alkali to neutralise its non-volatile acids, an amount corresponding to the presence of 1·44 gm. of lactic acid. Its volatile acids were much smaller, but dog's liver showed an

[1] Hahn, *Ber.*, 33, p. 3555, 1900.
[2] Magnus-Levy, *Hofmeister's Beitr.*, 2, p. 261, 1902.

inverse relationship, and formed much more volatile acids than non-volatile. The ratio of V to N-V was invariably low in ox liver autolyses, and high in dog liver autolyses. The data

Tissue.	Non-volatile Acids.	Volatile Acids.	V÷N-V.
Ox Liver, 1 day aseptic . . .	16·0	2·8	·18
„ 9 days „ . . .	19·1	4·8	·25
Dog's Liver, 1 day aseptic . .	2·2	8·9	4·0
„ 6 days „ . . .	2·7	18·0	6·7

show that most of the acid formation occurred during the first twenty-four hours. Another experiment showed that very little occurred in the first six hours, hence there must have been a rapid disintegration between these two periods.

In comparison with aseptic autolyses, antiseptic ones occur extremely slowly; so much so that there may be less decomposition in six months than in a single day of aseptic autolysis. The following experiment is a case in point:—

Tissue.	Non-volatile Acids.	Volatile Acids.
Ox Liver, 1 day under aseptic conditions . .	16·0	2·8
„ 2½ months with Chloroform . .	4·3	·95
„ 2½ „ with Toluol . . .	7·7	1·6
„ 6 „ „ . . .	8·3	2·0

In addition to these acids, the autolysing liver gave off a good deal of gas. In one experiment 45 gm. of rabbit's liver, under aseptic conditions, gave off 100 c.c. of gas in two days. Two-thirds of this gas was CO_2, and the remainder hydrogen. This considerable evolution of hydrogen suggests bacterial action, and Magnus-Levy realised that micro-organisms may have been present which were not revealed by his cultures. The fact that Lane-Claypon and Schryver[1] observed scarcely any more proteolysis when minced liver was kept in saline at 37° for twenty-four hours without an antiseptic than when it was kept in presence of toluol supports this view of bacterial infection.

[1] Lane-Claypon and Schryver, *Journ. Physiol.*, 31, p. 169, 1904.

On the other hand, Levy found that the acids formed in the aseptic autolyses were of the same character as those in the antiseptic ones, and so it looks as if they were both formed in the same way, viz. by enzyme action.

This autolytic acid formation is by no means confined to the liver, as the following data show :—

Organ.	Duration of Autolysis.	Non-volatile Acids.	Volatile Acids.
	Months.		
Spleen of Ox	5	7·6	5·6
Muscle of Horse	5	11·5	3·3
Salivary gland of Ox	4	3·6	3·5
Thymus of Ox	5½	3·0	1·6
Heart muscle of Calf	5	5·6	·6
Kidney of Dog	6	2·6	1·0
Testis of Bull	5	1·6	·6
Lymph glands of Ox	4½	1·3	1·2
Pancreas of Dog	6	1·4	·8
Lung of Calf	4½	1·4	·6
Ovary of Ox	4½	·8	·2

These tissues were kept under antiseptic conditions at 38°. It will be seen that only spleen and muscle developed as much acidity as ox liver.

The origin of these acids is very uncertain. Neumeister[1] and Asher and Jackson,[2] arguing more especially from the great excretion of lactic acid observed by Minkowski in geese with extirpated liver, think that the lactic acid is formed from proteins. Magnus-Levy, in common with many other physiologists, attributes their origin to carbohydrates. He found that during the aseptic autolysis of dog and ox liver a good deal of the glycogen and sugar disappeared, and that the actual loss corresponded fairly well with the increase of acidity. In making the calculation, he assumed that the acids formed were all produced from lactic acid, one molecule of acetic acid arising from one molecule of lactic acid, and one of butyric acid from two molecules of lactic acid, just as they seem to be in acetic and butyric fermentations. They could scarcely have been formed from the higher fatty acids of the liver fat, as Siegert[3] has shown

[1] Neumeister, *Lehrbuch d. physiol. Chem.*, 2nd ed., p. 313.
[2] Asher and Jackson, *Zeit. f. Biol.*, 41, p. 393, 1901.
[3] Siegert, *Hofmeister's Beitr.*, 1. p. 114, 1902.

that during liver autolysis the quantity of these acids remains unchanged.

The lactic acid present in fresh tissues is almost always the dextro-rotatory acid, whilst Levy found that that formed by autolysis is chiefly the inactive form. At least in antiseptic autolyses only 10 per cent. of it consisted of the dextro acid, but in aseptic autolyses as much as 40 per cent. was of this kind. Mochizuki and Arima[1] allowed minced bulls' testes to digest at 38° with toluol and chloroform, and they found that the dextro acid was the only one formed. The testes contained ·045 per cent. of the acid originally, and after two to fifteen days' autolysis it increased to ·126 to ·231 per cent. Also Kikkōji[2] observed a considerable formation of dextro acid in the autolysis of ox spleen. From 500 gm. of the fresh organ he isolated ·8 gm. of lactic acid. It will be remembered that the lactic acid produced by the action of an acetone preparation of *B. Delbrückii* on glucose was found by Buchner and Meisenheimer to be the inactive body, hence one must conclude that intracellular enzymes exist which can give rise to both forms of the acid.

[1] Mochizuki and Arima, *Zeit. f. physiol. Chem.*, 49, p. 108, 1906.
[2] Kikkōji, *ibid.*, 53, p. 415, 1907.

LECTURE V

OXIDISING ENZYMES

Oxygenases or aldehydases and their various activities. Peroxidases and their estimation. Doubtful enzymic nature of oxidases. Tyrosinases, laccase, and alcohol-oxidase. Catalases: their estimation, mode of action, and relation to functional capacity. Inorganic ferments. Respiration in dead animal and plant tissues, before and after disintegration, and its relation to respiratory enzymes. Intramolecular oxygen. Respiratory processes in biogens.

THE processes of oxidation which are continuously taking place in all living tissues are even more important than those of hydrolysis, in that they are chiefly responsible for the production of heat and other forms of energy. Though our knowledge of the subject is at present very fragmentary, it seems probable that these oxidations are brought about by intracellular oxidising enzymes. Hence the study of such enzymes is of great interest and importance. As long ago as 1863 Schönbein made a number of observations upon them, but since his time, and until the last few years, they have been almost completely neglected. Even now much of our information is very inexact and contradictory.

The oxidising enzymes have been separated by Bach and Chodat[1] into three main classes, and the scheme of classification suggested by them is generally accepted as a convenient and correct one. Members of the first class, the *true oxidases*, *oxygenases*, or *aldehydases*, possess the power, when in the

[1] Bach and Chodat, *Ber.*, 35, pp. 1275, 2467, 3943, 1902; 36, pp. 600, 606, 1756, 1903; 37, pp. 36, 1342, 2434, 3785, 3787, 1904; 38, p. 1878, 1905; 39, pp. 1664, 1670, 2126, 1906; 40, p. 230, 1907; *Biochem. Centralb.*, 1, pp. 417 and 457, 1903.

presence of oxygen, of oxidising aldehydes such as salicyl-aldehyde and formic aldehyde to their corresponding acids, or of oxidising tincture of guiacum resin to a blue colour. The second class of oxidases, the *peroxidases*, turn guiacum tincture blue only if hydrogen peroxide be present. The third class, the *catalases*, are probably not true oxidising enzymes at all, as they cannot turn guiacum blue either in presence or absence of hydrogen peroxide, but they have the power of liberating oxygen from hydrogen peroxide solution.

The guiacum test depends on the oxidation of guiaconic acid, $C_{20}H_{24}O_5$, to guiacum blue, $C_{20}H_{22}O_6$ (Doebner), so it is best carried out by using an alcoholic solution of pure guiaconic acid. Inorganic substances, such as iodine, chlorine, bromine, nitric acid, chromic acid, ferric chloride, copper salts, and many peroxides, likewise effect this oxidation. Besides this test several other colour reactions have been used for investigating the oxidases. Röhmann and Spitzer[1] showed that if a dilute alkaline solution of α-naphthol and paraphenylene-diamine are added directly to tissue pulp in presence of air, a blue-violet colour develops owing to the absorption of oxygen and the formation of indophenol. Pohl[2] found that extracts of the tissues gave a similar reaction. Bourquelot[3] observed that oxidases turn guiacol red. Kastle and Shedd[4] found that phenolphthalin is changed by them to phenolphthalein; that paraphenylene-diamine is changed to a dark brown colour, and that the colourless reduction products of indigotin and methylene blue are re-oxidised. Whether all these reactions are brought about by true oxidases, or can to some extent be induced by peroxidases and other bodies, has not yet been properly investigated. The evidence, such as it is, suggests that several of the different reactions are brought about by different enzymes, or that a number of oxidases exist. Thus Rosell[5] precipitated the oxidases from various tissue extracts by means of uranyl

[1] Röhmann and Spitzer, *Ber.*, 28, p. 567, 1895.
[2] Pohl, *Arch. f. exp. Path.*, 38, p. 65.
[3] Bourquelot, *C. R. Soc. Biol.*, 46, p. 896, 1896.
[4] Kastle and Shedd, *Amer. Chem. Journ.*, 26, p. 527, 1901.
[5] Rosell, "Dissertation," Strassburg, 1901, quoted from *Ergebnisse der Physiol.*, I., i., p. 233.

acetate, and investigated the action of solutions of the precipitates. In no case did they give the guiacum reaction, whilst in every case they liberated oxygen from hydrogen peroxide. To the salicylaldehyde and indophenol tests, however, they reacted very differently. Some extracts gave a positive result in both cases, others a negative result in both cases, and others one negative and one positive result, as the following data show: —

Tissue.	Aldehydase.	Indophenol Oxidase.
Salivary glands	+	+
Thymus	+	+
Spleen	+	+
Lungs	+	−
Brain	+	−
Suprarenal glands	+	−
Testis	+	−
Kidney	+	−
Lymph glands	+	−
Pancreas	−	+
Bone marrow	−	+
Mammary glands	−	−
Muscle	−	−

Abelous and Biarnès[1] found that the minced tissue of most organs contained aldehydase, but did not give the indophenol reaction. Again, Kastle and Shedd[2] found that extracts of sheep's liver and testis—which are known to contain aldehydase—did not give either the guiacum reaction or oxidise phenolphthalin to phenolphthalein. On the other hand, extracts of most vegetable tissues, such as potato and maize, did both, and there was a rough parallelism between the depth of the two reactions given by each tissue.

The aldehyde-oxidising power of the tissues was first investigated by Schmiedeberg,[3] who found that if oxygenated blood containing benzyl alcohol or salicylaldehyde were perfused through a freshly excised liver or lung, a small amount of the corresponding acid was formed. Jaquet[4] repeated these

[1] Abelous and Biarnès, *Arch. de Physiol.*, 1895, pp. 195 and 239.
[2] Kastle and Shedd, *loc. cit.*
[3] Schmiedeberg, *Arch. f. exp. Path.*, 14, pp. 288 and 379, 1881.
[4] Jaquet, *ibid.*, 29, p. 386, 1892.

experiments, and showed that even after death the tissues still retained their oxidative power. A lung previously frozen or kept with 2 per cent. phenol solution for forty-eight hours still possessed half or more of its original oxidising capacity. A kidney or half a lung of a horse was kept for twelve days in 75 per cent. alcohol, and was then perfused with saline or blood containing salicylaldehyde or benzyl alcohol for two-and-a-half to five hours at 37°. From ·032 to ·053 gm. of the corresponding acid was formed. Even after the organs were minced up they preserved a good deal of their activity. Two kidneys (of the horse) were minced, hardened with alcohol, and dried, and the product kept, with frequent shaking, with 1 litre of blood and 1 gm. of salicylaldehyde for twenty-four hours at 25° to 30°. The salicylic acid formed amounted to ·13 gm., whilst 1 kg. of horse muscle, similarly treated, gave only ·02 gm. of the acid. Again, the juice expressed from half a lung (horse) and kept for five hours at 35° to 40° with blood and salicylaldehyde, gave only ·023 gm. of salicylic acid. The oxidation was due at least in part to a soluble enzyme, as a filtered extract of two alcohol-hardened kidneys, when kept with the aldehyde, yielded ·012 to ·036 gm. of salicylic acid.

It will be seen that the salicylic acid formed was in every case very small in amount, considering the very large weight of lung or kidney taken. Abelous and Biarnès,[1] who used fresh tissues, obtained somewhat better results. They added 100 gm. of tissue to 1 litre of saline containing ·5 per cent. of Na_2CO_3 and 2 c.c. of salicylaldehyde, and kept the mixture for twenty-four hours at 38° in a current of air. The following amounts of salicylic acid were formed:—

Tissue.	No Antiseptic.	1 per cent. NaF.
	gm.	gm
Spleen	·252	...
Lung	·146	·142
Liver	·139	·139
Thyroid	·098	...
Kidney	·062	·077
Thymus	·061	·060
Suprarenal gland	·060	...
Testis	·023	...

[1] Abelous and Biarnès, *loc. cit.*

It will be seen that the oxidation was little if at all diminished by the presence of 1 per cent. of sodium fluoride. Three of the tissues worked with, viz. pancreas, muscle, and brain, effected no oxidation at all, either with or without NaF. It will be remembered that Rosell likewise found no aldehydase in pancreas and muscle. Blood contained a small amount of the enzyme, as 1 kg. of defibrinated ox blood, kept for twenty-four hours at 38° in a current of air with salicylaldehyde, formed ·176 gm. of salicylic acid, and 1 kg. of pig's blood formed ·060 gm. of acid. Abelous and Biarnès confirm Jaquet's conclusion that the oxidase is a soluble ferment, but they found, as he did, that a good deal of it is destroyed in the process of coagulating the tissue with alcohol, drying, and extracting it. Salkowski and Yamagiwa,[1] like Abelous and Biarnès, found that spleen tissue possessed the most active oxidasic power, whilst liver was only a little inferior. The kidney, on the other hand, formed only a tenth to a twentieth as much salicylic acid as the spleen. They did not find pancreas and muscle to be entirely lacking in enzyme, but pancreas formed only a twentieth to a hundredth as much salicylic acid as the spleen, and muscle not a hundredth as much. Zanichelli,[2] on the other hand, found that the pancreas possessed a good deal of oxidising power.

The oxidising action of the tissues on formic aldehyde was studied by Pohl.[3] It proved to be very weak, for an extract of 200 gm. of ox liver gave only ·0068 gm. of formic acid in one hour, and ·033 gm. in fifteen hours, whilst aqueous chloroform or sodium fluoride extracts had no oxidative power whatever.

The solubility and precipitability of aldehydase has been studied by Jacoby.[4] An extract of minced ox liver was fractionally precipitated by ammonium sulphate, and it was found that whilst very little of the enzyme was thrown down by 33 per cent. saturation with the salt, all of it was thrown down by 60 per cent. saturation. This precipitate could be dissolved up again in water, re-precipitated with dilute alcohol, and when re-dissolved again furnished an active oxidase solution.

[1] Salkowski and Yamagiwa, *Centralb. med. Wiss.*, 32, p. 913, 1894.
[2] Zanichelli, *Arch. di Farmacol.*, 3, p. 8.
[3] Pohl, *Arch. f. exp. Path.*, 38, p. 65.
[4] Jacoby, *Zeit. f. physiol. Chem.*, 30, p. 135, 1900.

Bach and Chodat[1] fractionally precipitated the enzymes in the juice of the fungus *Lactarius*, and they found that 40 per cent. alcohol threw down all the true oxygenase, whilst much of the catalase was still left in solution.

Peroxidases.—We have seen that true oxidases, though of wide distribution, are not present in all tissues, or if present, they are in such small amount that they cannot be demonstrated. Peroxidases, on the other hand, are stated by several investigators to be present in all living tissues. However, Loew[2] found that aqueous extracts of certain tobacco plants, though they had an energetic action on hydrogen peroxide, were unable to turn guiacum tincture blue in its presence. Kastle and Loevenhart[3] were unable to obtain the reaction with onion bulb: but as they point out, it is to be remembered that Hunger[4] showed that the reaction is sometimes masked by the presence of glucose and other powerful reducing substances.

The evidence of the almost universal presence of peroxidases is, in the case of animal tissues at least, to be accepted with reserve, because the guiacum and hydrogen peroxide test is given by hæmoglobin. As Czyhlarz and v. Fürth[5] point out, it is almost impossible to remove the hæmoglobin thoroughly from the tissues by perfusion, and hence the method is unreliable. A better test for peroxidase depends on its power of hastening the liberation of iodine from acidified potassium iodide solution in presence of hydrogen peroxide, for this reaction is not influenced by hæmoglobin. A quantitative measure of the action can be obtained by titrating the free iodine against sodium thiosulphate solution (Bach and Chodat). Even this iodine method is very imperfect, for the reaction may be completely stopped if much protein or other iodine-binding tissue constituents are present, and hence only a positive result is of significance. Czyhlarz and v. Fürth find that leucocytes, bone marrow, spleen, lymph glands, and spermatozoa give the reaction. The enzyme is contained in the cell constituents, and

[1] Bach and Chodat, *Ber.*, 36, p. 606, 1903.
[2] Loew, *Report* 68, *U. S. Dept. of Agriculture*, 1901.
[3] Kastle and Loevenhart, *Amer. Chem. Journ.*, 26, p. 539, 1902.
[4] Hunger, *Ber. d. bot. Ges.*, 19, p. 574.
[5] Czyhlarz and v. Fürth, *Hofmeister's Beitr.*, 10, p. 358, 1907.

not in the surrounding liquids, though it can be partially extracted from the cells by salt solutions. For the quantitative estimation of tissue peroxidases, Czyhlarz and v. Fürth use a spectrophotometric method, dependent on the oxidation of the leuco base of malachite green to the actual blue-green pigment. They find that with true tissue peroxidases the rate of oxidation is at first proportional to the time, but that as the reaction proceeds it slows down more and more, and after an hour or so stops altogether. The amount of oxidation so induced is directly proportional to the amount of enzyme present. Solutions of hæmatin also bring about the oxidation, but in their case the rate of oxidation is strictly proportional to the time throughout, and shows no tendency whatever to slow down. Also this hæmatin reaction is greatly affected by variation in the concentration of the catalysing pigment and the hydrogen peroxide, but only slightly by variation in the leuco base, whilst the peroxidase reaction is much more dependent on change in the concentration of the leuco base than on that of the peroxide. Buckmaster[1] has shown that hæmoglobin induces the reaction in the same way as hæmatin, but that hæmatoporphyrin and hæmatoidin do not. Hence the presence of iron in the molecule is essential, just as it is essential for the guiacum reaction.[2]

In that numerous inorganic oxidising agents can effect the conversion of guiaconic acid into guiacum blue, of salicylaldehyde into salicylic acid, and the other reactions held to indicate the presence of oxidases, it might reasonably be questioned whether these oxidases are enzymes at all, and are not merely unstable organic bodies, such as organic peroxides, which possess feeble oxidising power. The chief justification for classing them with the enzymes seems to lie in their extreme instability, and in the fact that their precipitability by salts roughly corresponds to that of other enzymes. On the other hand, they resist a considerably higher temperature than other enzymes, for Czyhlarz and v. Fürth found that peroxidase solutions could be heated nearly to boiling point without losing all their activity. Again, the oxidases do not seem to possess what is the most important and distinctive property of enzymes, viz. that of

[1] Buckmaster, *Journ. Physiol.*, 37, p. xi., 1908.
[2] Buckmaster, *ibid.*, 35, p. xxxv., 1907.

acting as a catalytic agent, or of being able to induce an indefinitely large amount of chemical change without themselves undergoing destruction. The extremely small production of salicylic acid from salicylaldehyde has already been commented on, and when we remember that some enzymes, such as invertase, can hydrolyse at least 100,000 times their weight of the substance upon which they are exerting their specific activity, we are forced to conclude, either that the tissues contain excessively small amounts of the oxidases, or that these bodies are not true enzymes in the ordinarily accepted sense. The latter conclusion is supported by the action of peroxidase on the malachite green base, for we saw that the final amount of oxidation effected by it was proportional to the amount of enzyme present, and that it very soon came to an end.

But what is the probable nature and mode of action of these pseudo-enzymes? Bach [1] considers that the oxidases of the blood are simply readily oxidisable substances which have a special capacity for forming peroxides. Kastle and Loevenhart [2] agree with him, and extend his view to the oxidases of the tissues. They think that organic peroxides, *i.e.* oxidases, are not present in the intact cells as such, but are in the form of readily oxidisable substances which in the presence of air or oxygen unite with the oxygen to produce the peroxide. These peroxides can then transfer their oxygen to other less readily oxidisable substances such as salicylaldehyde or guiaconic acid. A concrete instance of an organic peroxide of this character is found in benzaldehyde, for Baeyer and Villiger [3] have shown that this body, when exposed to atmospheric oxygen, is oxidised to benzoyl hydrogen peroxide, $C_6H_5.CO-O-OH$. Hence a mixture of benzaldehyde and water, in presence of air, turns guiaconic acid blue directly. Benzoyl hydrogen peroxide reacts with reducing agents such as indigo, guiaconic acid, etc., to form benzoic acid and an oxidation product, but as it is itself oxidised when acting as an oxygen carrier, its activity comes to an end. It is not a true catalytic agent, therefore, and Kastle and

[1] Bach, *Comptes Rendus*, 124, p. 951, 1897.
[2] Kastle and Loevenhart, *Amer. Chem. Journ.*, 26, p. 539, 1902.
[3] Baeyer and Villiger, *Ber.*, p. 1569, 1900.

Loevenhart think that the so-called oxidising enzymes of the tissues are peroxides of a similar non-catalytic character.

As regards the peroxidases, Kastle and Loevenhart suggest that their blueing action upon guiacum $+H_2O_2$ depends on the H_2O_2 first reacting with one or more organic substances present in the plant or animal extract to form an organic peroxide. This peroxide then oxidises the guiaconic acid to guiacum blue. A concrete instance of such a reaction is found in acetic peroxide, a highly unstable body which Baeyer and Villiger have prepared by the action of H_2O_2 on acetic anhydride. This body reacts with guiaconic acid to form guiacum blue and acetic acid.

Even if further research proves that the oxidases are true catalytic agents, they may still be of the nature of unstable organic peroxides, only they may have the property of taking up oxygen and passing it on to oxidisable substances without themselves undergoing further change.

In addition to the oxidases and peroxidases mentioned, several other oxidising enzymes have been recorded in animal tissues. It will be remembered that the liver contains an oxidase which can convert xanthin and hypoxanthin into uric acid. Whether this oxidation is the work of specific enzymes, or is dependent on the aldehydase of the liver, we do not know.

Tyrosinase.—Another oxidase, which is apparently specific, is the tyrosin-oxidising enzyme tyrosinase. This body was discovered by Bertrand[1] in plants in 1896, whilst two years later Biedermann[2] showed that a similar enzyme is present in the intestinal juice of the meal-worm (*Tenebrio molitor*). v. Fürth and Schneider[3] found it in the body fluids of certain Lepidoptera. They showed that the darkening which the hæmolymph undergoes on exposure to air is due to a tyrosinase. A preparation of the enzyme was obtained by precipitating the press juice of the pupæ with half-saturated ammonium sulphate. A solution of the precipitate in ·05 per cent. Na_2CO_3, when shaken in air with tyrosin solution, changed to a violet and then to a black colour, and finally yielded a precipitate of melanin. This

[1] Bertrand, *Bull. de la Soc. Chem.* (3), 15, p. 793, 1896.
[2] Biedermann, *Pflüger's Arch.*, 72, p. 105, 1898.
[3] v. Fürth and Schneider, *Hofmeister's Beitr.*, 1, p. 229, 1901.

melanin contained 13·7 per cent. of nitrogen, and smelt of indol when heated with caustic soda. In addition to its action upon tyrosin, tyrosinase gives the indophenol reaction and will convert other aromatic bodies such as pyrocatechin, hydroquinone and suprarenin into melanins. It does not give a typical guiacum reaction.

Tyrosinase seems to be widely distributed in the animal kingdom. It is present in the crayfish, in Cephalopods (*Sepia officinalis*) and in sponges, and Miss Durham[1] has shown that it is present in the skin of fœtal and new-born rats and rabbits. An aqueous extract of the ground-up skin, if mixed with tyrosin and a drop of ferrous sulphate to serve as activator, gradually takes on a dark colour and forms a black precipitate. An extract of the skin of red-skinned guineapigs gives under similar conditions an orange-coloured pigment.

Vegetable tyrosinase is probably as widely distributed as animal tyrosinase. Bertrand found that if the fungus *Russula nigricans* is boiled with alcohol and extracted with boiling water, some of its chromogen, which is a tyrosin-like body, passes into solution. If this solution is mixed with a cold-water extract of the fungus, it turns red and subsequently black. If the extract is added to a solution of tyrosin, it acts in the same way and changes it first red, then black, and then forms a black precipitate. The conversion is dependent on the presence of air, and an absorption of oxygen accompanies it. The juice expressed from the roots of the beet and the dahlia, and from the tubers of the potato, likewise contains tyrosinases which convert the tyrosin-like bodies present into melanins and act similarly upon added tyrosin. Bourquelot[2] found that tyrosinase also acts upon numerous other aromatic substances such as the cresols, resorcinol, guiacol, thymol, and naphthol.

Vegetable Oxidases.—Most of the observations recorded above, except those in the last paragraph, concern the oxidases of animal tissues. Similar classes of enzymes are found also in vegetable tissues. True oxidases, capable of blueing guiacum without the addition of hydrogen peroxide, are more widely

[1] Durham, *Proc. Roy. Soc.*, 74, p. 310.

[2] Bourquelot, *Comptes Rendus*, 123, pp. 315 and 423, 1896; *Bull. de la Soc. Mycol. de France*, 13, p. 65, 1897.

distributed in plants than in animals. They are especially abundant in potatoes, and in apples and other fruit, just beneath the skin. They give the indophenol reaction, and oxidise phenolphthalin to phenolphthalein, but their action on salicylaldehyde has scarcely been investigated at all. In addition to the oxygenases and peroxidases mentioned, other specific oxidising enzymes have been described. One of these, *laccase*, is the first oxidase definitely recognised as such. In 1883 Yoshida[1] showed that the production of lacquer varnish from the sap of the lac tree of South-East Asia is dependent on this enzyme. The fresh lac juice is nearly white, but on exposure to air it rapidly changes to brown, then black. It dries with a brilliant black lustre, owing to its chief constituent, urushic acid, $C_{14}H_{18}O_2$, being converted into oxyurushic acid, $C_{14}H_{18}O_3$, by the action of the laccase. Bertrand[2] has shown that laccase can oxidise hydroquinone to quinone, and pyrogallol to purpurogalline, and can act similarly upon other polyphenols. He finds that enzymes with the same action as laccase are present in many other plants, such as the roots of the beet, carrot, and turnip: in the potato, apple, and pear: in the vegetative parts of clover and asparagus; in the flowers of *Gardenia*, and in many fungi, whilst Rey-Pailharde has found it in germinating seeds. Arguing from its distribution, one would imagine that laccase is one and the same enzyme as the guiacum-blueing oxygenase, and indeed Bertrand used the guiacum test to some extent for its identification. On the other hand, the activity of laccase seems to be associated with the presence of manganese. Its ash always contains traces of the metal—sometimes over 1 per cent. of it—and Bertrand found that the activity of an enzyme preparation is proportional to the manganese present.

Laccase is distinct from tyrosinase, but Bourquelot[3] found that all of the many fungi examined by him contain both enzymes in varying proportions. If a solution of the two

[1] Yoshida, *Journ. Chem. Soc.*, 43, p. 472, 1883. See also, Reynolds Green's *Soluble Ferments and Fermentation*, from which this brief account is mainly drawn.

[2] Bertrand, *Comptes Rendus*, 118, p. 1215, 1894; 120, p. 266, 1895; 121, p. 166, 1895; 122, p. 1132, 1896; 123, p. 463, 1896; 124, pp. 1032 and 1355, 1897.

[3] Bourquelot, *loc. cit.*

enzymes be heated to 70°, the tyrosinase is destroyed, whilst the laccase remains.

Alcohol-oxidase.—More interesting, perhaps, than any of the above described vegetable oxidases is the alcohol-oxidase of acetic acid fermentation. Buchner, working in conjunction with Meisenheimer[1] and subsequently with Gaunt,[2] endeavoured to show that this fermentation is the work of an oxidising enzyme. To obtain sufficient bacteria of acetic acid fermentation, beer wort to which 4 per cent. of alcohol and 1 per cent. of acetic acid had been added was used as a culture medium, and was inoculated with bacteria from pure cultures. The bacteria were separated by centrifugalisation, dried fifteen to twenty hours on a porous clay plate, rubbed up for fifteen minutes with 20 volumes of pure acetone, washed two or three times with ether on a filter, and dried *in vacuuo* over sulphuric acid. A sample of 20 gm. of this "Acetondauerpräparat" was placed in a litre flask with about 250 c.c. of 4 per cent. ethyl alcohol, 8 c.c. of toluol, and excess of calcium carbonate to neutralise the acid formed, and was kept in a current of air for three days at 28°. The best experiment showed a yield of 4 gm. of acetic acid per 100 gm. of dried bacteria (equivalent to about 1000 gm. of moist living bacteria), so the oxidation was small compared with that effected by living bacteria. Press juice from the bacteria had no oxidising power at all, so Buchner and Gaunt suggest that the oxidase may have been destroyed during the process of extraction, or that it is an insoluble body which does not pass out in the juice. But unfortunately one is compelled to entertain still a third alternative, viz. that the oxidation effected by the acetone preparation was due solely to the presence of living bacteria. Buchner and Gaunt tested their preparations upon sterile beer wort, and found that a considerable proportion of them contained living bacteria. They think that these living bacteria formed only a very small fraction of the whole, but there is no exact evidence on the point. Living bacteria, if placed in beer wort in presence of toluol, produced very much less acetic acid than in absence of toluol, but they still formed five or ten times more acid than an equal amount of

[1] Buchner and Meisenheimer, *Ber.*, 36, p. 634, 1903.
[2] Buchner and Gaunt, *Annalen*, 349, p. 140, 1906.

acetone bacteria. This shows, according to Buchner and Gaunt, that the acetone treatment destroys a good deal of the oxidase, but one might reasonably argue that it killed all but a fifth or a tenth of the bacteria, and that this small fraction was responsible for the whole of the acetic acid formation. Until more adequate proof has been afforded, therefore, we cannot accept the existence of an alcohol-oxidase as proven.

Catalases.—The third class of oxidising enzymes, the catalases, is the most widely distributed of all, for every animal and vegetable tissue or fluid which gives the peroxidase reaction with guiacum and hydrogen peroxide can also decompose the peroxide, whilst certain of them which give the latter reaction are unable to effect the former. The presence of catalase but absence of peroxidase in aqueous extracts of certain tobacco plants has already been noted. Senter[1] prepared a solution of a "hæmase" from blood which energetically decomposed hydrogen peroxide, but did not give the peroxidase reaction.

Schönbein[2] found that all the enzymes tested by him had the power of blueing guiacum *plus* hydrogen peroxide, and also of decomposing this peroxide, so he thought that both these properties were specific for enzymes. Jacobson[3] showed that this was not the case, for emulsin, if heated to 69°, loses its power of decomposing hydrogen peroxide, but still retains its action on amygdalin. Also the catalases are very unstable, so that fresh tissue extracts when kept for some time, or extracts of dried tissues, lose their power of decomposing H_2O_2, though still preserving their other activities.[4] If a large excess of H_2O_2 be added to an enzyme solution, its catalytic power upon the peroxide may be lost, but not its specific fermentative power.

The first attempt at quantitative comparison of the catalytic power of various animal tissues was made by Spitzer,[5] who placed 1 or 2 gm. of the fresh blood-free tissue in a flask with 10 c.c. of 2 or 3 per cent. H_2O_2 solution, and measured the rate

[1] Senter, *Proc. Roy. Soc.*, 74, p. 201, 1904.
[2] Schönbein, *Journ. f. prakt. Chem.*, 89, p. 323, 1863.
[3] Jacobson, *Zeit. f. physiol. Chem.*, 16, p. 340.
[4] Kobert and Fischer, *Pflüger's Arch.*, 99, p. 123, 1903.
[5] Spitzer, *ibid.*, 67, p. 615, 1897.

of evolution of oxygen. In all cases the oxygen came off most rapidly at first, and then gradually dwindled down, but the initial rate of evolution was much greater with some tissues than with others. Spitzer arranged them in the following order, according to their catalytic activity: blood, spleen, liver, pancreas, thymus, brain, and muscle. Of these organs the thymus and pancreas were taken from the ox, and the remainder from the dog. Several other investigators have repeated these observations, and have found a more or less similar order of activity. For the various tissues of the calf Abelous[1] found it to be liver, kidney, pancreas, spleen, heart, lung, thymus, brain, and striped muscle, whilst for those of the ox Rosenbaum[2] gives it as liver, pancreas, spleen, muscle, and brain. Much more complete series of observations have been made by Battelli and Stern.[3] They point out that Spitzer used far too much tissue for the peroxide taken, and in their experiments they took care that there was always a considerable excess of peroxide. The oxygen liberated in ten minutes by the addition of 5 c.c. of dilute emulsion of the fresh tissue

Animal.	Liver.	Kidney.	Blood.	Spleen.	Lung.	Heart.	Muscle.	Brain.
Guineapig . .	5800	480	490	350	260	99	34	20
Rabbit . . .	370	390	460	148	98	65	16	10
Dog	624	210	51	33	52	15	9	7
Cat	1390	180	540	150	250	32	21	12
Pigeon . . .	1480	340	14	66	...	...	...	...
Adder . . .	460	200	1390	...	355	237	59	19
Frog . . .	3570	483	60	130	195	31	7	53
Fish (*Leuciscus*) .	2540	...	55	...	...	25	6	29

to 30 c.c. of 1 per cent. H_2O_2 was measured, and the data in the table show the volumes of gas liberated per decigram of tissue. In most animals the liver possessed the greatest catalytic activity, and guineapig's liver set free no less than 5800 c.c. of oxygen per decigram, or in the actual experiment, ·001 gm. of liver liberated 58 c.c. The next most active guineapig tissue,

[1] Abelous, *C. R. Soc. Biol.*, 1899, p. 328.
[2] Rosenbaum, *Festschrift f. Salkowski*, Berlin, 1904.
[3] Battelli and Stern, *Arch. di Fisiol.*, 2, p. 471, 1905.

the kidney, liberated only a twelfth as much oxygen, whilst the least active tissue, the brain, liberated only about a three-hundredth as much. The relative order of activity of the several tissues examined differs somewhat in different species of animals, though in every case but two the liver proved to be the most active organ, whilst muscle and brain were always the least active. The greatest abnormalities were found in the liver of the rabbit, which was less active than the kidney and blood, and in the blood and tissues of the adder. The adder's blood liberated nearly three times more oxygen than any mammalian blood, and one hundred times more than pigeon's blood, whilst adder's lung and cardiac muscle liberated considerably more oxygen than the corresponding tissues in other animals. It might be thought that these erratic values were largely dependent on chance variations, or experimental error, but Battelli and Stern say that for animals of the same species the values obtained for each tissue are remarkably constant, and do not differ from one another by more than 30 per cent. Again, Lesser[1] has quite independently obtained equally wide differences with the blood of various animals. He found that 100 c.c. of ·3 per cent. solution of blood decomposed the following weights of hydrogen peroxide in ten minutes at 38°: rabbit, ·754 gm.; dog, ·157 gm.; pigeon, ·015 gm.; frog, ·011 gm.

It will be seen that the tissues of warm-blooded animals were no more active than those of cold-blooded ones, and that allied species of animals such as the rabbit and the guineapig showed no more resemblance to one another than to any other vertebrate animal. It might seem hopeless, therefore, to attempt any deductions as to the possible functions of catalase from such apparently incompatible data as these; but other experimental results obtained by Battelli and Stern indicate that there is a close relationship between the catalytic power of a tissue and its functional activity. The table shows the oxygen liberated by the tissues of guineapigs of various ages, and it will be seen that with the growth of the embryo and of the new-born guineapig for the first week after birth, there was an enormous increase in the catalytic activity of the liver and

[1] Lesser, *Zeit. f. Biol.*, 48, p. 1, 1906.

kidney, and a slight one in that of the other tissues. As already mentioned in a previous lecture, I observed a similar large increase in the ereptic power of the liver and kidney of guinea-pigs, rabbits, and cats during intra-uterine growth and for the first week of post-natal existence. I also found a distinct though less considerable increase in the ereptic power of brain and muscle.

	Liver.	Kidney.	Blood.	Spleen.	Lung.	Muscle.	Brain.
5 gm. embyro .	350	30	...	...	...	...	12
17 „ „ .	450	70	...	230	...	15	12
36 „ „ .	780	110	390	290	...	19	12
Fœtus at term .	1200	150	405	300	180	24	16
2-day Guineapig .	2000	240	510	330	360	24	14
7-day „ .	5400	410	480	285	360	24	16
Adult „ .	5800	480	490	350	260	34	20

Starvation of rats for four to eight days, or of frogs for several months, did not influence the catalytic power of the tissues, neither did fatal poisoning with KCN or phosphorus: but doses of phosphorus which were not sufficient to kill, but which produced fatty degeneration of the liver, caused the catalytic power of this organ to dwindle to half or a third its normal value. On the other hand, the kidney, blood, lung, muscle, and brain showed a considerable increase in their catalytic power. Jolles and Oppenheim[1] found that the catalytic power of human blood is greatly diminished in certain diseases such as tuberculosis, nephritis, and carcinoma.

In its physical properties catalase appears to resemble other enzymes. Senter[2] found that it could be precipitated from laked blood by alcohol, and extracted from the precipitate with water. From 30 to 40 per cent. of the total enzyme originally present was thereby obtained in solution, and this solution, if kept at 0°, preserved its activity for some weeks. Loew[3] used ammonium sulphate as a precipitating agent, and from aqueous extracts of leaves of the tobacco plant he isolated two catalases; one, α-catalase, was soluble only in dilute alkalis, and was

[1] Jolles and Oppenheim, *Münchener med. Woch.*, N. 47, 1904.
[2] Senter, *Zeit. f. physik. Chem.*, 44, p. 257, 1903.
[3] Loew, *loc. cit.*

decomposed by it with formation of β-catalase. It appeared to consist of catalase in combination with a nucleoprotein, whilst β-catalase had the properties of a proteose.

There is no good proof that the nucleoprotein α-catalase is a genuine entity. More probably it consisted of certain nucleoprotein constituents of the plant tissues which had adsorbed some catalase. A similar explanation may hold for Spitzer's[1] results. Spitzer stated that the nucleoproteins of the liver, pancreas, kidney, testis, and blood decomposed hydrogen peroxide as energetically as the tissues themselves, whilst acids, alkalis, heat, and protoplasmic poisons as potassium cyanide and hydroxylamine had an enfeebling or arresting action on these nucleoproteins just as they had on the tissues themselves. But Spitzer did not purify his nucleoprotein preparations very thoroughly, and in all his experiments he added such a large excess of nucleoprotein to the hydrogen peroxide that the oxygen liberated may have been due entirely to adsorbed catalase. Spitzer pointed out that these nucleoproteins contain iron, and he suggested that this iron plays an essential part in the oxidation phenomena of tissues and extracts. However, Senter showed that his blood catalase contained no iron.

The mode of action of catalase has been discussed at length by a number of investigators, but lack of adequate experimental data precludes any finality in their conclusions. It is important, in the first place, to decide whether catalase is a true oxidising ferment or not. Loew thinks that it is, as he found that his preparations of β-catalase, obtained from tobacco leaves and the juice of the poppy seed, were able to oxidise hydroquinone to quinone, though they did not give any of the other oxidase reactions. Shaffer[2] thinks that this quinone formation was undoubtedly due to the presence of some enzyme other than catalase, for he and Buxton[3] found that embryonic and adult animal tissues always contained catalase, but frequently possessed no power of oxidising hydroquinone. Again, we know that anaërobic organisms such as intestinal worms and certain micro-organisms contain catalase, which in these instances

[1] Spitzer, *Pflüger's Arch.*, 67, p. 615, 1897.
[2] Shaffer, *Amer. Journ. Physiol.*, 14, p. 299, 1905.
[3] Shaffer and Buxton, *Journ. Med. Research*, 8, p. 543, 1905.

cannot possibly exert an oxidising function. On the other hand, Ewald[1] found that if a small quantity of blood catalase is added to hæmoglobin solution, the mixture is reduced by ammonium sulphide two or three times more rapidly than if no catalase is added. Hence he thought that the catalase acted as an oxygen carrier from the oxyhæmoglobin to the ammonium sulphide. Czyhlarz and v. Fürth[2] repeated this experiment, but they found that the addition of unboiled catalase extract to hæmoglobin and ammonium sulphide did not lead to any more rapid reduction than the addition of boiled extract, hence they conclude that the catalase has no direct oxidising action.

Inorganic ferments.—It has long been known that hydrogen peroxide is decomposed by many inorganic substances, as well as organic, and Schönbein[3] found that hydrocyanic acid not only prevented its decomposition by various animal and plant enzymes, but also inhibited the action of platinum black upon it. Hence he concluded that the catalytic influence of the organic and inorganic substances is of similar character. In recent years Bredig[4] and his co-workers have brought forward fresh evidence in support of this view, and have shown that close analogies exist between the action of colloidal gold and platinum upon hydrogen peroxide, and that of catalase. So much so that Bredig speaks of these inorganic colloids as "inorganic ferments," and refers to the inhibitory effect upon their action produced by traces of hydrocyanic acid, sulphuretted hydrogen and other substances as a "poisonous" influence. He finds that just as HCN is the strongest poison for blood catalase, so it is the strongest poison for colloidal platinum. For instance, the presence of ·0014 mg. of HCN per litre was sufficient to reduce the activity of a colloidal platinum preparation to half its original value, whilst other blood poisons such as cyanogen iodide, corrosive sublimate, phosphorus, and carbonic oxide, behaved similarly towards the platinum. Kastle and Loeven-

[1] Ewald, *Pflüger's Arch.*, 116, p. 334, 1907.

[2] Czyhlarz and v. Fürth, *loc. cit.*

[3] Schönbein, *Zeit. f. Biol.*, 3, p. 140, 1867.

[4] Bredig and v. Berneck, *Zeit. physik. Chem.*, 31, p. 258, 1899; Bredig and Ikeda, *ibid.*, 37, p. 1, 1901; Bredig and Reinders, *ibid.*, 37, p. 323, 1901; Bredig, "Anorganische Fermente," Leipsig, 1901.

hart[1] wholly disagree with this analogy, and say that when it holds at all it is a mere coincidence. They find that many substances act altogether differently on the two orders of catalysers. Potassium bromide considerably retards the action of liver catalase upon hydrogen peroxide, and it similarly retards the action of finely divided platinum. On the other hand, it accelerates the action of finely divided copper and iron, but completely inhibits the action of silver and thallium. Hydroxylamine doubles the action of silver upon the peroxide, does not influence the action of platinum, but reduces that of catalase to a twenty-fifth its original value. Thio-urea accelerates the action of liver catalase, but reduces that of silver and platinum to a tenth their normal values.

Kastle and Loevenhart say that the effect of any particular substance upon an inorganic catalyst can be explained, at any rate in the majority of cases, on purely chemical grounds. The inhibitory substance may form a thin insoluble protective film over the surface of the catalytic metal.

The mode of action of catalases is probably of an essentially different character from that of inorganic catalysts. As Shaffer[2] points out, the oxygen liberated by catalase from hydrogen peroxide is only molecular oxygen, whilst inorganic catalysts such as platinum black can absorb molecular oxygen, or oxygen from H_2O_2, and render it active. The difference in the character of the oxygen is shown by the fact that colloidal platinum can turn guiaconic acid blue (Bredig[3]), and liberate iodine from potassium iodide (Liebermann[4]), whereas catalase cannot. Hence Liebermann's hypothesis, that the action of catalase upon H_2O_2 takes place in the following two stages—

$$\text{Ferment} + H_2O_2 = \text{Ferment} \cdot O + H_2O$$
$$\text{Ferment} \cdot O + H_2O_2 = \text{Ferment} + O_2 + H_2O$$

—is probably incorrect, though it is very likely that the action of platinum black takes place in accordance with a similar scheme, which may be represented thus—

$$Pt + H_2O_2 = PtO + H_2O$$
$$PtO + H_2O_2 = Pt + O_2 + H_2O.$$

[1] Kastle and Loevenhart, *Amer. Chem. Journ.*, 29, pp. 397 and 563, 1903.
[2] Shaffer, *Amer. Journ. Physiol.*, 14, p. 299, 1905,
[3] Bredig, "Anorganische Fermente," Leipsig, 1901.
[4] Liebermann, *Pflüger's Arch.*, 104, p. 670, 1904.

As regards the function of the catalases in the living tissues we are entirely in the dark. It has been suggested by Loew that in the processes of tissue respiration hydrogen peroxide is constantly being formed, and as it is a violent poison, catalase must be present to prevent its accumulation. This hypothesis is improbable for several reasons. In the first place, hydrogen peroxide is not a violent poison, as Bach and Chodat[1] have cultivated certain plants in a medium containing ·68 per cent. of it. But even if it were a poison, it could not be formed at all in anaërobic organisms, yet these organisms, as stated above, contain catalase just like aërobic ones. Then if hydrogen peroxide were formed during respiration, one would expect the catalase content of a tissue to be a measure of its respiratory activity. But a reference to the table on p. 128 shows that this cannot be the case. The respiratory activity of guineapig's liver cannot be sixteen times greater than that of rabbit's liver, or 290 times greater than that of guineapig's brain. In fact, it is possible that the catalytic power of a tissue is a measure of its reducing power rather than of its oxidising power. Pozzi-Escot[2] considers that catalase is identical with the "hydrogenase" of Rey-Pailharde,[3] and called by him philothion, from its power of converting free sulphur into sulphuretted hydrogen. Bach and Chodat[4] do not admit the identity of these two ferments, but the fact of its being suggested shows how supremely ignorant we are of the function of catalases. That such a function does exist, seems to be proved by the variations in the catalytic power of the tissues with functional activity. Of course it is possible that the power of decomposing H_2O_2 is a chance property of certain organic substances formed in the tissues, but even if this is the case it is important for us to elucidate the meaning of the reaction, as it would give us a convenient measure of the amount of such hypothetical substances in the tissues, and the variations they undergo in different phases of cellular activity.

[1] Bach and Chodat, *Biochem. Centralb.*, 1903, p. 417.

[2] Pozzi-Escot, *Comptes Rendus*, 134, p. 66, 1902; *Amer. Chem. Journ.*, p. 29, 517, 1903.

[3] Rey-Pailharde, *Comptes Rendus*, 106, p. 1683, and 107, p. 43, 1888; *C. R. Soc. Biol.*, 46, p. 455, 1894.

[4] Bach and Chodat, *loc. cit.*

Tissue Respiration.—Before discussing the probable or possible part played by oxidases and peroxidases in tissue respiration, it will be well to adduce evidence to show that the respiration is probably due to enzymes of some kind or other. I endeavoured[1] to obtain some proof of it by measuring the gaseous metabolism of a convenient organ, viz. the mammalian kidney, under various abnormal conditions. The gaseous exchange was determined by perfusing it continuously with oxygenated Ringer's solution for about twelve hours. The gases were boiled off *in vacuuo* from samples of the saline solution before and after perfusion, and analysed, and so the intake of oxygen and output of CO_2 were measured. A kidney kept for twenty-four hours at a temperature of $-8°$ to $-1°$ (whereby it was frozen hard) had about a third the gaseous metabolism of normal kidneys, whilst another kidney frozen to $-8°$ and thawed again three times on three successive days had about half the normal metabolism. In that Pictet[2] found that frogs survived exposure to a temperature of $-28°$, it might reasonably be held that this freezing did not kill the tissues, so in another series of experiments the kidneys were heated for half an hour to various temperatures up to 60° before they were perfused. It has been found by Halliburton[3] that the kidney contains a cell globulin coagulating at 50° to 55°, and it has been shown by Brodie and Richardson,[4] and by myself,[5] that muscle when gradually heated undergoes shortening in several steps, at temperatures which roughly correspond with those at which the muscle proteins undergo coagulation. So presumably exposure of a kidney to a temperature of over 50° would cause some protein coagulation, or would precipitate one of the constituents of the protoplasm of the cells, and would thereby destroy the vitality of these cells. Yet I found that a kidney retained about a fifth of its normal gaseous metabolism after being heated to 53°, whilst kidneys heated to 55° and 60° retained a tenth of their metabolism. Again, perfusion of a kidney with

[1] Vernon, *Journ. Physiol.*, 35, p. 53, 1906.
[2] Pictet, *Rev. Scient.*, 52, p. 577, 1893.
[3] Halliburton, *Journ. Physiol.*, 13, p. 810, 1892.
[4] Brodie and Richardson, *Phil. Trans. Roy. Soc.*, 191 B, p. 127, 1899.
[5] Vernon, *Journ. Physiol.*, 24, p. 239, 1899.

1 per cent. sodium fluoride or 1 per cent. arsenious acid solution caused the tissue respiration to fall to less than a third the normal during the next few hours, but it did not absolutely stop it even in three days. In all of these experiments the respiratory quotient kept at about ·85, or the ratio of CO_2 production to oxygen absorption was the same as in the normal living kidney.

Upon plants numerous observations have been made by Palladin[1] and his co-workers. The vegetable organs under observation were killed by freezing them at a temperature of $-20°$ to $-3°$ for twenty hours. A current of hydrogen was then drawn over them at room temperature, and the CO_2 outflow determined. From etiolated leaves of the vetch the CO_2 came off at the rate of ·028 gm. per hour per 100 gm. of tissue during the first four hours; at ·009 gm. per hour during the next four hours, and at ·0024 gm. per hour during the next fifteen hours. Palladin suggested that this CO_2 was formed by a hypothetical "carbonase" enzyme. On replacing the hydrogen by an air current, a fresh outflow of CO_2 began, and continued at the rate of ·007 to ·005 gm. per hour for the next forty hours or more. Its formation was supposed by Palladin to be due to an oxidase enzyme. The amounts of CO_2 evolved from germinating wheat and etiolated vetch leaves varied between the following limits. They are calculated for 100 gm. of plant substance:—

Plant.	In Hydrogen Current (Carbonase).	In Air Current (Oxidase).
Germinating Wheat	1·025 to 1·282 gm.	0
Etiolated Leaves of Vetch . . .	·100 to ·185 gm.	·142 to ·245 gm.

No direct observations upon the oxygen intake were made in these particular experiments, but on keeping other (previously frozen) etiolated vetch leaves in a closed volume of air over mercury, and analysing samples from time to time, it was found that the oxygen was absorbed in rough proportion

[1] Palladin, *Zeit. f. physiol. Chem.*, 47, p. 407, 1906; Palladin and Kostytschew, *ibid.*, 48, p. 214, 1906; Maximow, *Ber. d. Deutsch. bot. Ges.*, 22, p. 904; Tscherniajew, *ibid.*, 23, 1905; Palladin, *ibid.*, 23, 1905; 24, 1906; Krasnosselsky, *ibid.*, 1906.

to the CO_2 given out. The actual respiratory quotients obtained varied from 2·0 to ·3.

It might naturally be asked if there is any proof of the existence of these carbonases and oxidases which are supposed by Palladin to be responsible for the CO_2 formation. No attempts were made to isolate them, and in fact Palladin lays stress on the fact that any injury to the anatomical structure of the dead plant acts destructively on the activity of the respiratory enzymes. Hence the proof of their existence depends entirely on this output of CO_2 from the dead plants. The CO_2 evolved in a hydrogen current is very probably due to an intracellular zymase, whilst that subsequently evolved in an air current may with considerable probability be referred to an oxidase. Palladin found that there was a further output of CO_2 if the plant leaves were rubbed up in a mortar with pyrogallic acid solution, and the mixture kept in an air current: but probably this CO_2 is not of enzymic origin, as Stoklasa, Ernest, and Chocenský[1] found that if vegetable tissues such as the leaves and root of the beet were slowly dried, powdered, and then heated to 150° for fourteen hours, so as to destroy all the enzymes, they still yielded about as much CO_2 when treated with pyrogallic acid as the fresh tissues did.

The great instability of the respiratory enzymes is shown by Palladin and Kostytschew's observations upon peas. As was stated in the previous lecture, living seeds of the pea and other plants form CO_2 and alcohol when kept in a hydrogen atmosphere. But living seeds kept in air show no accumulation of alcohol, *i.e.* they are able to oxidise it as it is formed. Frozen pea seeds, on the other hand, accumulate a considerable amount of alcohol in presence of full oxygen supply, so the freezing seems to destroy their respiratory oxidases more than their zymase. For instance, 200 frozen pea seeds, kept at 20° for 98 hours in a current of air, formed 1·482 gm. of CO_2 and 1·013 gm. of alcohol, or in proportion of 100 to 68. An equal quantity of frozen seeds kept for the same time in a current of hydrogen, formed ·775 gm. of CO_2 and ·553 gm. of alcohol, or in proportion of 100 to 71.

Palladin's conclusion that the respiration of plants is

[1] Stoklasa, Ernest, and Chocenský, *Zeit. f. physiol. Chem.*, 50, p. 303, 1907.

dependent on their structural integrity might be thought to apply to animal tissues as well, but this is very far from being the case. Thunberg[1] pounded up the muscles of one leg of a frog in a mortar for 15 to 30 minutes, and compared their respiration with that of the uninjured muscles of the opposite limb by means of his microrespirometer. During the first hour, at room temperature, the pounded muscles absorbed 3·07 c.c. of oxygen per 100 gm., whilst the uninjured muscles absorbed 5·94 c.c., or nearly twice as much. In succeeding hours the oxygen absorption of the uninjured muscles remained fairly constant, whilst that of the injured muscles rapidly deteriorated, so that in the fourth hour the former absorbed 4·86 c.c. of oxygen, and the latter 1·22 c.c., or a fourth as much. Lussana[2] crushed fresh tissues of the rabbit in a metallic sieve, and found that 100 gm. of liver, kept in a closed vessel of air at a temperature of 35° to 40° for four hours, absorbed 39·5 c.c. of oxygen and gave out 44·8 c.c. of CO_2, whilst muscle under similar conditions absorbed 12·2 c.c. of oxygen and gave out 8·7 of CO_2.

Much more striking are the results obtained by Battelli and Stern.[3] These observers minced up the tissues finely, and put 40 gm. of them with 100 c.c. of blood or other liquid in a 600 c.c. flask full of oxygen. The flask was kept in a water-bath at 38°, and was rapidly shaken by mechanical means. After half an hour or an hour the change in the volume of the gas in the flask was measured and a sample of it was analysed. The following data show the volumes of oxygen absorbed by 100 gm. of tissue in half an hour. The organs were removed from the dog and minced within 20 minutes of death :—

Kidney . . .	242 c.c.	Brain	82 c.c.
Heart muscle . .	194 ,,	Pancreas . . .	76 ,,
Skeletal muscle . .	184 ,,	Thyroid . . .	26 ,,
Liver	176 ,,	Spleen	19 ,,

As a rule the liver was somewhat more active than skeletal muscle. Otherwise these data correspond with the average results. It will be seen that they show an extremely large

[1] Thunberg, *Hammarsten's Festschrift*, Upsala, 1906.
[2] Lussana, *Arch. di Fisiol.*, 3, p. 113, 1906.
[3] Battelli and Stern, *Journ. de physiol.*, 9, pp. 1, 34, 228, and 410, 1907.

oxygen absorption, much larger, in fact, than that found in living animals. Regnault and Reiset and others found that the dog absorbs 700 to 1300 c.c. of oxygen per kilogram per hour, *i.e.* 35 to 65 c.c. per 100 gm. per half-hour, or very much less than the majority of these minced tissues. Again, Barcroft[1] found that individual organs of the dog, perfused with oxygenated blood under normal conditions, absorb the following volumes of oxygen per 100 gm. per half-hour when in a resting condition; pancreas, 150 c.c.; kidney, 90 c.c.; muscle of leg, 6 c.c. It would seem, therefore, that the mechanical injury to the tissues for the time being heightens their oxygen absorption powers considerably. It increases their CO_2 production in similar proportion, for the quotients obtained varied, as a rule, from ·7 to 1·4. After the first few minutes, the gaseous metabolism rapidly diminished, and in some cases ceased altogether after an hour or so. For instance, minced horse muscle, placed in saline, absorbed oxygen at the following rates per *hour:* 195 c.c. during the first 10 minutes; 240 c.c. in the next 20 minutes; 70 c.c. in the next 30 minutes; and 67 c.c. in the next 30 minutes. Some rabbit's muscle ceased absorbing oxygen after an hour and some horse's liver almost ceased absorbing after 40 minutes. But the results are extremely variable and irregular, and the method used by Battelli and Stern is undoubtedly a rough one and liable to considerable error. When the minced tissue and blood were shaken up with air instead of oxygen, the oxygen absorption was diminished by about 50 per cent. in the case of muscle, but only by about 20 per cent. in the case of other tissues. Again, the oxygen absorption was much smaller if the minced tissue were shaken up with saline instead of with blood. For instance, ox muscle in saline absorbed 50 c.c. of oxygen per hour: in a mixture of 2 of saline and 3 of blood, 132 c.c. per hour, and in blood only, 177 c.c. per hour. These differences were not due to any appreciable absorption of oxygen by the blood itself, but were presumably owing to the hæmoglobin of the blood acting as a more efficient oxygen carrier to the tissues.

Other observations were made upon the vitality of the tissues after death of the animal. Muscle of the dog, kept at

[1] Barcroft, *Biochem. Journ.*, 1, p. 6. 1906.

0° for seventy minutes after death, and then minced and placed in 1 per cent. disodium phosphate solution, absorbed 196 c.c of oxygen in half an hour, whilst muscle kept eighteen hours at 0° before mincing absorbed 58 c.c. Some of the same muscle kept for seventy minutes at 30° before mincing, absorbed 247 c.c. of oxygen, and when kept for seven hours at 30°, it absorbed only 6 c.c. Antiseptics greatly diminished the respiratory powers of the tissues. For instance, some rabbits' liver, when placed in blood and sodium phosphate solution, absorbed 118 c.c. of oxygen in half an hour, but when 1 per cent. of NaF was added it absorbed 23 c.c.; with ·01 per cent. KCN it absorbed 21 c.c.; and with ·02 per cent. of arsenite, only 6 c.c.

As Battelli and Stern point out, the minced tissues used in their experiments undoubtedly contained many intact and still living cells, and hence one is not justified in assuming their respiration to be entirely the work of respiratory enzymes. Still it is very remarkable that the gaseous metabolism should have been so large. The experiments, when repeated, should be made with tissues minced to various degrees of fineness, and their respiration should be measured over considerably longer periods. Also it would be of great interest to measure the gaseous metabolism of the expressed juice of tissues, free of all solid constituents, with a view to determining if respiratory enzymes are soluble bodies. Battelli and Stern[1] found that if freshly minced muscle were shaken up for five minutes with 1½ volumes of water or saline, and the extract were quickly strained off through a linen cloth, neither it or the solid residue of minced muscle had much respiratory activity when kept in a flask of oxygen on the water-bath; but if extract and residue were mixed, the mixture had more than three times the gaseous metabolism of either single constituent. For instance, 30 gm. of the solid residue of minced ox diaphragm muscle, mixed with 10 to 70 c.c. of extract, absorbed about 30 c.c. of oxygen in half an hour. The experimental results were very irregular, but a careful repetition and extension of them by more exact methods may be of great value to us in elucidating the mechanism of tissue respiration. As they stand, they seem to show that respiration is chiefly carried out by the interaction of

[1] Battelli and Stern, *Journ. de Physiol.*, 9, p. 737, 1907.

a soluble constituent of the tissues with some insoluble constituent of the cellular framework. But it is impossible to make such a deduction with any safety, as the extraction period of the minced muscle was so short.

Though in most observations on gaseous metabolism the respiratory quotient is found to approach unity, there is plenty of evidence to show that the CO_2 output is not directly dependent upon a contemporary oxygen intake. As long ago as 1803 Spallanzani showed that a frog continued to exhale CO_2 even when entirely deprived of oxygen. Pflüger,[1] and subsequently Aubert,[2] found that frogs kept in nitrogen containing no trace of oxygen continued to give out CO_2 for some hours at a rate but little inferior to that exhibited by frogs kept in air. They gave it out rapidly and for a brief period if kept at a high temperature, and slowly and for a long period if kept at a low one; but the total volume of CO_2 exhaled was practically the same in all cases, amounting to about 200 c.c. per kilogram body weight. Thunberg[3] found that a snail, *Limax agrestis*, when kept in a nitrogen atmosphere, exhaled more than 1100 c.c. of CO_2 per kilogram, whilst the mealworm, *Tenebrio molitor*, exhaled about 1000 c.c. The writer[4] found that the mammalian kidney, when perfused with boiled (oxygenless) saline, gave out about 100 c.c. of CO_2 per kilogram.

It is generally assumed that this CO_2 is formed at the expense of a supply of intramolecular oxygen which is stored up in the tissues, though as Winterstein[5] and the writer[6] point out, there is at present no completely satisfactory proof of its existence. But admitting the probability of its presence in the tissues, in what form is it stored up? Verworn,[7] arguing from the fact that increased functional activity of muscles and other organs has no effect whatever on nitrogenous metabolism, though it may increase their CO_2 output and oxygen intake

[1] Pflüger, *Pflüger's Arch.*, 6, p. 43, 1871; 10, p. 251, 1875; 14, p. 5, 1878.
[2] Aubert, *ibid.*, 26, p. 293, 1881.
[3] Thunberg, *Skand. Arch. f. Physiol.*, 17, p. 133, 1905.
[4] Vernon, *Journ. Physiol.*, 35, p. 53, 1906.
[5] Winterstein, *Zeit. f. allgem. Physiol.*, 6, p. 315, 1907.
[6] Vernon, *Sci. Progress*, 2, p. 160, 1907.
[7] Verworn, *Arch. f.* (*Anat. u.*) *Physiol.*, 1900, Suppl. p. 152; also, "Die Biogenhypothese," Jena, 1903.

tenfold, supposes that only non-nitrogenous groupings or side-chains of the "biogen molecules" of the protoplasm are concerned in the ordinary processes of tissue respiration. He suggests that these side-chains may be carbohydrate groupings of an aldehyde character, that the intramolecular oxygen is stored up in the biogens in the form of an NO_2 grouping attached to a benzene ring, and that one atom of oxygen from the NO_2 oxidises the $-CHO$ and $-CHOH$ groups of the carbohydrate molecule to CO_2 and water. This hypothetical NO_2 compound is comparable to the organic peroxide structure which Kastle and Loevenhart attribute to the oxidases of tissue extracts, and though at present neither they or Verworn have any direct experimental evidence in support of their views, it is quite possible that the tissue oxidases may ultimately prove to be compounds of this nature which have broken away from the cellular protoplasm in which they functioned as oxygen carriers.

The hypothesis that the respiratory processes of tissues are carried out by non-nitrogenous side-chains is supported by some curious and unexpected results which I obtained when investigating the gaseous metabolism of the mammalian kidney by the previously mentioned method. I found that the protoplasm is in a state of such instability that it is liable to undergo sudden disintegration, whereby as much as 9 to 17 per cent. of the total protein present in the kidney tissues is washed out during the course of an eleven-hour perfusion. Yet the respiratory powers of these kidneys were just as great as those of other kidneys in which there had been little or no tissue disintegration. The proof is not a complete one, however, as in all experiments the gaseous metabolism had dwindled to a half or less of its initial value by the end of the perfusion, and hence it is possible that the protein which broke away from the tissues consisted in part of the no longer functioning respiratory side-chains.

The carbohydrate-like nature of the side-chains concerned in tissue respiration is supported by evidence quoted in the previous lecture. We saw that the tissues, either alone or in combination with one another, had considerable glycolytic power. The existence of an enzyme of alcoholic fermentation, though not properly substantiated for animal tissues, seemed fairly well

established for vegetable ones, whilst Magnus-Levy showed that the liver and other tissues, when kept under aseptic conditions, rapidly formed considerable quantities of lactic and other acids. Though there is no actual proof that these acids were formed from carbohydrate, probability is all in favour of it. It is probable that the lactic acid and other intermediate bodies formed by the decomposition of carbohydrates and other substances in the tissues are subsequently oxidised to CO_2 and water by an intracellular oxidase or peroxide which takes up oxygen from the blood and transfers it to them. In the absence of an oxygen supply, these intermediate products accumulate more and more, whilst the CO_2 into which they would normally have been oxidised rapidly decreases. Thus Fletcher and Hopkins[1] found that absolutely fresh frog's muscle contained only a trace of lactic acid (·015 per cent. or possibly less), but if it were placed in an atmosphere of hydrogen or nitrogen, the acid gradually increased till by the time the muscle passed into rigor mortis it amounted to ·24 to ·40 per cent. A muscle which had accumulated a certain amount of acid as the result of fatigue or insufficient oxygen supply was able to oxidise and destroy it, or at least a part of it, if placed in an oxygen atmosphere. This oxidation may have been the work of an intracellular oxidase, but if so the enzyme is a very unstable body; for it was found that chopped-up muscle, when kept in oxygen, is unable to oxidise and destroy its lactic acid. However, the oxidising power of the tissues can be destroyed by less violent methods than mechanical disintegration, for I found that if a kidney were perfused with saline containing ·06 to ·10 per cent. of lactic acid, or ·005 to ·025 per cent. of free ammonia, it somewhat rapidly lost its oxygen-absorbing power, but it still more rapidly lost its CO_2-producing power, so that after four to eight hours' perfusion its respiratory quotient dropped to ·46 to ·36: *i.e.* only half the usual proportion of oxygen absorbed was being used to oxidise tissue constituents to CO_2.

It will be seen that at present we are supremely ignorant as to the connection of the oxidases and peroxidases with tissue respiration. Many of the isolated experimental results above described are of great interest in themselves, and they suggest

[1] Fletcher and Hopkins, *Journ. Physiol.*, 35, p. 247, 1907.

that the connecting links may be discovered in course of time. But much further investigation will first be required, not only to elucidate these links, but to sweep away a good deal of the inexact and careless experimental work with which this particular problem is especially encumbered.

LECTURE VI

THE CONSTITUTION AND MODE OF ACTION OF ENZYMES

Preparation of pure pepsin. Its protein-like nature. Influence of proteins on stability of enzymes. Precipitability of enzymes. Relation of rennin to pepsin, trypsin, and other proteolytic enzymes. Adsorption of enzymes and of dyes. Slight diffusibility of enzymes. Optical activity of enzymes. Chemical combination of enzyme with substrate. Velocity of enzyme action, and its deviations from law of mass action.

SUFFICIENT evidence has been adduced in previous lectures to show that many if not most of the chemical processes of living tissues are dependent upon enzymes, and hence if we are ever to understand the inner mechanism of these processes, it is essential for us to understand the chemical constitution and mode of action of enzymes. Though a large amount of work has been done upon both of these problems, the positive results obtained in answer to the former one have hitherto been very limited. And this for two reasons. Firstly, the enzymes are such extremely unstable bodies that it is impossible to purify them without destroying a great deal of their activity; and secondly, we have no absolute criterion as to how much of the product obtained is actual enzyme, and how much impurity. In attempting to isolate an enzyme from a solution, therefore, it is important that the methods of purification should be as gentle as possible, and that at each stage of the purification or concentration a quantitative estimation should be made of the total amount of enzyme present, so as to determine how much of it, if any, has been destroyed. In fact, the ideal method would be to follow that used by Madame Curie in isolating radium from pitchblende, when the unknown element was

traced by its radio-activity, and by various purification processes obtained in greater and greater concentration, till the preparation of maximum radio-active power was found to be the pure radium salt. Probably such a method would be impossible with enzymes, unless a temporary stability could be artificially induced, but there seems to be no other way of proving the purity of an enzyme preparation.

Practically all the attempts hitherto made to isolate a pure enzyme have been upon exoenzymes, but we have every reason for thinking that the endoenzymes closely resemble these bodies both in chemical and physical properties, and that what holds for the one class holds almost equally well for the other. Of the proteolytic exoenzymes, pepsin and to a less extent trypsin have received the most attention, but I do not intend to describe in detail the elaborate methods used by the earlier observers such as Brücke[1] and Kühne to isolate these enzymes, as they could by no possibility have yielded anything like a pure product. Brücke, for instance, allowed pig's stomach to digest itself for several days in presence of phosphoric acid—whereby a very large amount of the enzyme must have been destroyed — and then threw down a precipitate of calcium phosphate by the addition of lime water. The colloidal pepsin was adsorbed by this precipitate and carried down with it, but so was much of the protein impurity. The precipitate was dissolved in dilute HCl, and a second precipitate thrown down by the addition of more lime water. Each of these precipitations and re-solutions would undoubtedly destroy a large amount of the enzyme, though Brücke did not attempt to determine whether this was the case or not. The pepsin was then precipitated still a third time with cholesterin, and after various washings the cholesterin was extracted with ether, and a small quantity of slimy substance was left. This, when dissolved in ·1 per cent. HCl, was able to digest a flake of fibrin in about an hour; but a few drops of it diluted with 5 c.c. of ·1 per cent. HCl dissolved fibrin equally quickly, so it must undoubtedly have contained some impurity which checked its activity when in concentrated solution. The solution did not seem to contain a trace of protein, as it failed to give a cloud

[1] Brücke, *Sitzungsb. d. k. Akad. d. Wiss. Wien.*, 43, p. 601, 1861.

with nitric acid, tannin, or mercuric chloride. Hence Brücke concluded that pepsin cannot be a protein body.

It is probable, though there are no quantitative data bearing on the point, that the method of producing an inorganic precipitate in an enzyme solution, in the hope that it will carry down the enzyme with it but not the impurities, is a futile one, and calculated to destroy a larger proportion of enzyme than it removes of impurity. Salting out with ammonium sulphate or other salt is probably a much better method, as it is less violent, whilst fractional precipitation with alcohol may in some cases be a useful method, though it should always be accompanied by quantitative enzyme estimations.

The methods used by recent workers for the isolation of pure pepsin do not suffer from many of the disabilities of the older ones. In the first place, it is possible to obtain a very active pepsin solution containing but few of the protein and other impurities which must be present in every extract of gastric mucous membrane. This is done by giving a fictitious repast to a dog in which œsophageal and gastric fistulæ have been made in accordance with Pawlow's method. In this way several hundred cubic centimetres of pure gastric juice are obtained, uncontaminated by any food material. Schoumow-Simanowsky[1] found that if this juice were cooled to 0°, a fine powdery precipitate was thrown down which seemed to consist of pure pepsin. Saturation with ammonium sulphate threw down a precipitate of very similar composition, as can be seen from the data given in the table below. These figures show that the precipitated product had the composition of proteins with the addition of ·80 to 1·17 per cent. of chlorine (present as hydrochloric acid). No proof was furnished of the identity of the precipitates with pepsin, other than similarity of composition and their very great digestive activity.

Nencki and Sieber[2] endeavoured to obtain a purer pepsin from gastric juice by the method first adopted by Pekelharing.[3] This depends on the fact, first noted by him, that if an extract of pig's stomach containing pepsin and hydrochloric acid be

[1] Schoumow-Simanowsky, *Arch. f. exp. Path.*, 33, p. 336, 1894.
[2] Nencki and Sieber, *Zeit. f. physiol. Chem.*, 32, p. 291, 1901.
[3] Pekelharing, *ibid.*, 22, p. 233, 1897.

dialysed for fifteen to twenty hours, all but ·02 per cent. of the HCl dialyses away, and a precipitate is thrown down. This precipitate, when dissolved in dilute hydrochloric acid, digests proteins very vigorously, and was thought by Pekelharing to be pepsin mixed with a variable amount of a phosphorus-containing body as impurity. Nencki and Sieber found that the product thrown down from dialysed gastric juice had an elementary composition similar to that found by Schoumow-Simanowsky, except that it contained only about half as much chlorine. It also contained ·073 to ·148 per cent. of phosphorus, and ·11 to ·18 per cent. of iron. Hence they concluded that pepsin is a nucleoprotein containing iron, and that it is bound up with HCl. They found by qualitative tests that small quantities of lecithin were present, and they thought that it was in chemical combination with the pepsin, and not merely mixed with it.

Pepsin prepared by	C.	H.	N.	S.	Cl.
Cooling Juice to 0° (Schoumow-Simanowsky)	50·73	7·23	...	·98	1·09
Precipitating with $(NH_4)_2SO_4$ „	50·37	6·88	14·77	1·29	·84
Dialysing Juice (Nencki and Sieber) . .	51·26	6·74	14·33	1·50	·475
„ „ (Pekelharing) . . .	51·99	7·07	14·44	1·63	·49
Precipitating with $(NH_4)_2SO_4$ (Pekelharing)	52·16	7·09	14·70	1·83	...

Nencki and Sieber were mistaken in ascribing such a highly complex structure to pepsin, as Pekelharing,[1] who subsequently prepared pepsin by the same method, obtained a colourless product which contained only ·01 per cent. of phosphorus, whilst by ammonium sulphate precipitation he obtained a pepsin containing no phosphorus whatever. It could not possibly be a nucleoprotein, therefore, and the small quantity of phosphorus found by Nencki and Sieber, and by himself in his earlier preparations, must have been due to impurity. He took care to filter his gastric juice before dialysing, which Nencki and Sieber did not do, and this may have removed small quantities of nucleoprotein-containing mucus and cell debris shed from the gastric mucous membrane. Pekelharing did not make any analyses of the iron content of his preparations, but there can

[1] Pekelharing, *Zeit. f. physiol. Chem.*, 35, p. 1, 1902.

be little doubt that it is an impurity, even though Nencki and Sieber found it was always present in fairly constant amount. Supposing the pepsin molecule contained only a single atom of iron, its molecular weight would come to about 50,000 if only ·11 per cent. of iron were present; but such a high molecular weight is improbable, as in its physical properties pepsin closely resembles other proteins of lower molecular weight. It is precipitated by half saturation with ammonium sulphate, and is coagulated and destroyed at about the same temperature as they are. Thus a solution heated to 65° for two minutes becomes faintly opalescent, and loses a little of its digestive power; heated to 70° it becomes opalescent, and loses most of its digestive power; heated to 75° it forms a white cloud, and loses the whole of its digestive power.[1]

Hence we may provisionally conclude that pepsin is a protein body containing neither iron or phosphorus. Friedenthal[2] found that it contains a pentose group, capable of yielding an osazone with phenylhydrazine, and Pekelharing confirms this conclusion. Pekelharing found almost exactly the same percentage of chlorine as did Nencki and Sieber, and he agrees with them in thinking that this chlorine is a constituent of the protein molecule. When the pepsin was well washed with 96 per cent. alcohol, ·29 per cent. of this ·48 per cent. of chlorine present was removed. The enzyme at the same time completely lost its digestive activity, so perhaps this is definitely dependent upon the presence of the chlorine.

If pepsin is a protein-like body, how is it that Brücke and other observers have obtained purified preparations of the enzyme, which according to them showed considerable digestive activity but which gave few or none of the protein reactions? The explanation of this apparent contradiction probably depends on the fact that the digestion test for pepsin is a very much more delicate one than any known protein test, and in their efforts to purify their pepsin these observers obtained a solution too dilute to give aught but the digestion test. Hofmeister[3] states that the biuret test is not yielded by

[1] Pekelharing, *Zeit. f. physiol. Chem.*, 22, p. 242, 1897.
[2] Friedenthal, *Arch. f. (Anat. u.) Physiol.*, 1900, p. 186.
[3] Hofmeister, *Zeit. f. physiol. Chem.*, 2, p. 291, 1879.

a 1 in 10,000 protein solution. Nitric acid gives a cloud with a 1 in 20,000 solution, and Millon's test also gives a positive result at this dilution, but not at still greater dilutions. Potassium ferrocyanide and acetic acid give a distinct cloud with a 1 in 50,000 solution, but not at double the dilution. Tannic acid, phosphotungstic acid, and Brücke's reagent are the most delicate tests of all, as they give a reaction with a 1 in 100,000 solution of protein. However, Pekelharing states that ·000,001 gm. of his pepsin, dissolved in 6 c.c. of ·2 per cent. HCl, dissolved a flake of fibrin in some hours, so sixty times this quantity of enzyme would yield a digestive solution of considerable activity. Yet even then it would form only a 1 in 100,000 protein solution, or would give none of the usual protein reactions except the last three cited.

The evidence concerning the preparation of pure pepsin has been described at some length, as it seems to me that in this one case the probabilities are in favour of a nearly pure product having been obtained. The method of preparation is so simple that there can have been very little destruction of enzyme during its progress. The same cannot be said of the processes employed with any other enzyme, and hence it will not be necessary to describe them in detail. Trypsin, for instance, has probably never been obtained in a condition of even approximate purity. It has hitherto been prepared from pancreatic extracts, which must contain a large amount of protein impurity, as well as erepsin and other intracellular enzymes. Pancreatic juice would afford no more suitable a source of the enzyme, as it contains large amounts of protein and of other enzymes, and moreover it would have first to be activated by the addition of enterokinase.

For a long time past it has been urged that enzymes are nucleoprotein bodies, and though this cannot be true of pepsin, there is no reason why it should not hold for other enzymes. In the case of fibrin ferment, for instance, a large amount of evidence has been adduced in favour of this view, for it is found that snake venoms and extracts of tissues and of alcohol-coagulated blood serum contain nucleoproteins, and can also coagulate fibrinogen. But no adequate proof has yet been

afforded that the clotting is due to the nucleoprotein and not to a thrombin adsorbed by it.

Again, it is possible, though not probable, that some enzymes are not protein bodies at all. O'Sullivan and Tomson[1] isolated the invertase from aqueous solutions of yeast by the gradual addition of 70 per cent. alcohol. After standing two days, the precipitate thrown down was washed in alcohol of 47 per cent. concentration, and was then re-dissolved in water. By no means all of the precipitate passed into solution, but almost all of the invertase must have done so, as only 12 per cent. of the original inverting power was lost. The enzyme preparation contained some ash, most of which could be removed by dialysis, but otherwise O'Sullivan and Tomson think that it was fairly pure. It contained 46·4 per cent. of carbon, 6·63 per cent. of hydrogen, and 3·69 per cent. of nitrogen, and may have been a combination of protein with a carbohydrate. However, there is no proof that the enzyme was at all pure, as on attempting to purify it by fractional alcohol precipitation it quickly lost its activity. In fact, the purer the enzyme the sooner did it lose its activity and the more readily was it destroyed by alcohol. The stability of the enzyme was found to be greatly increased by the presence of cane-sugar. When a solution of the purified enzyme was quickly heated to 45°, and cooled, no less than 70 per cent. of its activity was lost, and when heated to 50°, 98 per cent. was lost. But when cane-sugar was present it could be heated to 60° without losing any activity at all, and on heating to 70° it lost only 66 per cent. of its activity. This protective influence of the sucrose is due, in all probability, to the enzyme entering into a loose combination with it.

Purified preparations of invertase and other enzymes are more unstable than impure ones, largely because of the absence of protein impurity. Any excess of alkali, acid, salt, or other substance present which may tend to act harmfully upon the enzyme then reacts and enters into loose combination with the protein, and to a corresponding degree spares the protein-like enzyme. Thus Falk[2] found that the retardation exerted

[1] O'Sullivan and Tomson, *Journ. Chem. Soc. Trans.*, 1890, p. 834.

[2] Falk, *Virchow's Arch.*, 84, p. 119, 1881.

by a small amount of hydrochloric acid upon the amylolytic action of saliva was much diminished if peptone were present. Chittenden and Ely[1] found that peptone would also prevent sodium carbonate from exerting its retarding action upon saliva to a large extent. Langley and Edkins[2] observed that the destructive action of sodium carbonate upon pepsin is diminished by the addition of proteins. This was probably due, they thought, to the alkali combining with the protein, "for the greater the amount of sodium carbonate present, the greater must be the amount of protein to lessen appreciably the destruction." Biernacki[3] found that proteoses and peptones exert a considerable protective influence upon trypsin, for the trypsin of pancreatic extracts was completely destroyed when kept for five minutes at 50° with from ·25 to ·5 per cent. of Na_2CO_3, but if proteoses or peptones were added, it had to be heated to 60° before undergoing a similar rapid destruction. Even at 37° trypsin is rapidly destroyed by sodium carbonate, for I found[4] that in one hour ·4 per cent. Na_2CO_3 destroyed from 55 to 75 per cent. of the enzyme in a fresh preparation. If any protein, proteose, or peptone were present, however, it protected the enzyme in proportion to its concentration. Thus in presence of ·4 per cent. of protein I found[5] that on an average 45 per cent. of the trypsin was destroyed in an hour; with 1 per cent. of protein, 27 per cent. was destroyed; with 2 per cent. of protein, 12 per cent. was destroyed; and with 4 per cent. of protein, only 7 per cent. was destroyed. When no protein at all was added, 56 per cent. of the trypsin was destroyed in the hour. Protein decomposition products could protect the trypsin as well as protein itself, and in fact the protective influence of a substance seemed to depend entirely upon its power of neutralising the sodium carbonate. Amino acids such as glycin, taurin, leucin, aspartic acid, and hippuric acid acted as efficiently as proteins, whilst creatin, urea, glucose, and maltose, which are unable to combine with alkali, exerted

[1] Chittenden and Ely, *Amer. Chem. Journ.*, 4, p. 107, 1882; *Journ. Physiol.*, 3, p. 327, 1882.

[2] Langley and Edkins, *Journ. Physiol.*, 7, p. 371, 1886.

[3] Biernacki, *Zeit. f. Biol.*, 28, p. 49, 1891.

[4] Vernon, *Journ. Physiol.*, 27, p. 269, 1901; and 28, p. 448, 1902.

[5] Vernon, *ibid.*, 31, p. 346, 1904.

no protective effect whatever. It seems highly probable, therefore, that trypsin, pepsin, and other enzymes resemble proteins in being able to act as weak acids when in alkaline solution, and as weak alkalis when in acid solution. The protective influence of proteins and their decomposition products upon them is chiefly one of mass action, as they can likewise act as pseudo-acids or pseudo-bases, and combine with the destructive alkali or acid present.

Though we are justified in regarding enzymes as protein-like bodies, it is probable that they differ as much from one another in their chemical constitution and physical properties as do the proteins from which they may be derived. For instance, they differ a good deal in their precipitability. Danilewsky[1] found that if collodion solution were added to pancreatic extracts, a precipitate was thrown down which when extracted with water gave a solution containing trypsin but no diastase. The filtrate from the precipitate, however, contained a large amount of diastase, but only a little trypsin. These results have been partially confirmed by Lossnitzer.[2] Also Dastre[3] found that trypsin is only slightly soluble in 50 per cent. alcohol, whilst pancreatic diastase is slightly soluble in 65 per cent. alcohol. I have carried out[4] systematic fractional precipitations of diluted glycerin extracts of pancreas with alcohol, and have estimated the amounts of tryptic, rennetic, and diastatic enzymes still left in solution in the filtrates, and those present in solutions of the precipitates. As can be seen from the data in the table, increasing strengths of alcohol threw down increasing but proportionate amounts of trypsin and rennin, so that the ratio of the one ferment to the other was constant both in the filtrates and in the solutions of the precipitates. In confirmation of previous observers, I found that the diastase was much less readily precipitable. The filtrate from a mixture of 1 part of extract with 2·5 parts of alcohol contained more than four times as much diastase, relative to trypsin, as the original extract. In every case the

[1] Danilewsky, *Virchow's Arch.*, 25, p. 279, 1862.
[2] Lossnitzer, *Arch. d. Heilk.*, 5, p. 556, 1864.
[3] Dastre, *Arch. de Physiol.*, 8, p. 120, 1896.
[4] Vernon, *Journ. Physiol.*, 29, p. 302, 1903.

processes of precipitation and re-solution of the enzymes destroyed about 45 per cent. of the trypsin and rennin present, but with the greater strengths of alcohol (2 to 3 vols.) no less than 76 to 86 per cent. of the diastatic enzyme was destroyed. Other experiments showed that with more concentrated glycerin extracts the addition of three volumes of alcohol might destroy 99 per cent. of the diastatic enzyme in three hours. Hence fractional alcohol precipitation is obviously not

	Filtrate.					Precipitate dissolved in Water.			
	Tryptic Value.	Rennetic Value.	R÷T.	Diastatic Value.	D÷T.	Tryptic Value.	Rennetic Value.	R÷T.	Diastatic Value.
Glycerin Extract alone . .	31·7	95·5	3·0	89·3	2·8	...	...	...	...
1 of Extract to ·8 of Alcohol .	29·0	79·6	2·7	81·2	2·8	2·0	...	...	4·6
1 „ 1 „ .	26·6	72·7	2·7	79·8	3·0	4·2	...	...	6·4
1 „ 1·5 „ .	20·8	55·9	2·7	68·0	3·3	8·6	17·2	2·0	7·4
1 „ 2 „ .	9·4	28·3	3·0	56·6	6·0	15·2	31·3	2·1	7·7
1 „ 2·5 „ .	2·2	...	...	26·1	11·9	22·1	45·2	2·0	9·7
1 „ 3 „ .	·0	...	...	2·6	...	24·6	52·1	2·1	12·2

a suitable method for the purification of these particular enzymes.

The fact that trypsin and rennin have identically the same precipitability by alcohol raises a point of some interest, and one which has received a good deal of attention of recent years. Nencki and Sieber[1] found that the pepsin they obtained by dialysing gastric juice had a milk-coagulating function as well as a peptic one, and they suggested that one and the same molecule might have different enzyme actions. Just as Ehrlich in his side-chain theory considers that these chains have different configurations and functions, so they think that the pepsin molecule may exert a hydrolytic function by one of its side-chains, and a milk-coagulating one by another. Still they admit that their pepsin-rennin preparation may have been a mixture, as they made no attempt to separate the two enzymes. However, Grützner,[2] and subsequently Winogradow,[3] found that

[1] Nencki and Sieber, *Zeit. f. Physiol. Chem.*, 32, p. 291, 1901.
[2] Grützner, *Pflüger's Arch.*, 16, p. 119, 1878.
[3] Winogradow, *ibid.*, 87, p. 120, 1901.

the quantities of peptic and of rennetic ferments in the gastric mucous membrane at various times after a meal run completely parallel. Pawlow and Parastschuk [1] fed dogs, in which a small side stomach had been made, with milk, meat, and bread, and found that the juice collected from the stomach during successive hours after a meal showed parallel changes in its protein-digesting and milk-coagulating power with the different diets. Also the acid juice, when kept in an incubator and tested from day to day, was found to deteriorate to an equal extent in respect of both these activities, and the same thing was observed when the juice was heated for a short time to a temperature of 50° to 60°. The same parallel between proteolytic and milk-coagulating power was observed with activated pancreatic juice. Hence Pawlow and Parastschuk conclude that both these activities are due to one and the same enzyme, and they show that Hammarsten was mistaken in supposing that he had been able to separate the pepsin and rennin in gastric juice.

It was stated by Bang [2] that the rennet ferment in extracts of calf stomach differed from the ferment in extracts of pig's stomach in that it was differently affected by the addition of calcium chloride and of alkali, and was more rapidly destroyed when heated to 70°. Gewin [3] found that these differences were due to the presence of impurities, and that the enzymes differed less and less from one another the more they were purified. He also found that minced coagulated egg albumin adsorbed pepsin and rennin equally from a solution of the two ferments, and that half saturation with ammonium sulphate precipitated them equally. Hence he agrees with Pawlow and Parastschuk as to the identity of pepsin with rennin.

On the other hand, I have made a number of observations upon pancreatic extracts which seem to show that Nencki and Sieber's hypothesis is the correct one, and that the proteolytic and milk-coagulating powers are due to different side-chains of a single enzyme molecule. Thus I found [4] that if dilute alcholic and saline extracts of pancreas were kept for some months and tested from time to time, their tryptic and rennetic

[1] Pawlow and Parastschuk, *Zeit. f. physiol. Chem.*, 42, p. 415, 1904.
[2] Bang, *Pflüger's Arch.*, 79, p. 425.
[3] Gewin, *Zeit. f. physiol. Chem.*, 54, p. 32, 1907.
[4] Vernon, *Journ. Physiol.*, 27, p. 269, 1901.

powers varied independently, so that at one time the rennetic power, relative to the tryptic, might be seven times greater than at another. As a rule the trypsin is more unstable than the rennin, and when in two experiments the minced pancreas was kept for twenty-five and sixty-three hours respectively before the addition of the extracting liquid, it was found that 83 and 75 per cent. respectively of the tryptic activity was lost, whilst in the first experiment only 30 per cent. of the rennetic power was lost, and in the second experiment, none whatever of it. Still the tryptic and rennetic enzymes of an extract, though they can vary independently and be to some extent destroyed independently, can never be separated from one another. We saw above that their precipitability by alcohol was identical, and I found[1] that if minced pancreatic tissue were fractionally extracted, they passed into solution at the same rate. The minced pancreas was shaken for one or two hours with twice its volume of the extracting medium (slightly diluted glycerin or dilute alcohol), and was then separated from it by centrifugalisation. Two more volumes were added, and these were separated in the same way after about twenty hours' extraction. The next extraction lasted four days, the next eight days, and the next twenty-five days. The amounts of trypsin, rennin, and diastase in these several extracts were estimated, and it was found that the ratio of rennin to trypsin was practically constant throughout. For instance, in the five glycerin extracts of a pig's pancreas, the rennetic values were 2·8, 2·4, 2·7, 3·1, and 3·1 times the respective tryptic values. On the other hand, the ratio of diastase to trypsin varied greatly, and in one experiment it sank from 3·9 : 1 for the first extract, down to ·34 : 1 for the last.

It seems probable that a rennetic enzyme invariably accompanies every proteolytic one, even though it may be impossible for it ever to exert its milk-coagulating power. Thus it is present in the stomach of fishes, and the pancreas of many animals : in the fruit, seeds, and leaves of many plants, and in many micro-organisms. Edmunds[2] found that it is present in small quantity in the liver, spleen, kidney, thyroid, thymus, lung, muscle, brain, small intestine, testis, and ovary, or is as uni-

[1] Vernon, *Journ. Physiol.*, 28, p. 448, 1902.
[2] Edmunds, *ibid.*, 19, p. 466, 1895.

versally present as β-protease and erepsin. It may not always be found in sufficient quantity to curdle milk, for I have shown[1] that the proteolytic ferment is antagonistic to the milk-curdling action, in that it tends to dissolve the curd as fast as it is formed. But the presence of the rennin can always be demonstrated by means of Roberts' "metacasein" reaction. Because of its universal occurrence, it seemed to me possible, if not probable,[2] that rennin is not a genuine ferment of functional significance, but is merely a by-product in the formation of proteolytic enzymes, which chances to possess the property of curdling milk.

The enzymes, though protein-like bodies, must evidently possess certain groupings which are not present in the inert protein molecule, and which confer upon them their lability and their catalytic powers. What these special groupings consist of we are entirely ignorant. Loew[3] suggests that they may be ketone groups, in that hydrocyanic acid, which readily forms additive compounds with ketones, paralyses the action of many enzymes. If the hydrocyanic acid is removed, the enzyme recovers its activity, so that its combination with the ketone groups must be a loose one, easily dissolved. Again, hydrazine and hydroxylamine, which in 1 per cent. solution at 40° destroy the activity of pepsin, trypsin, and diastase in two to four hours, likewise react readily with ketone groups to form hydrazones and oximes respectively. Loew thinks that the lability of enzymes is heightened by the presence of amino groups. Thus nitrous acid, which even in dilute solution readily acts upon amino groups, destroys enzymes very rapidly. Formaldehyde behaves similarly, though greater concentration is necessary. But in any case there can be but little doubt that enzymes, in virtue of their protein-like nature, contain amino groups.

The lability of some enzymes seems to be a variable factor, quite apart from the presence or absence of protein or other substances conferring stability upon them. As already mentioned, I found[4] that the tryptic enzyme in fresh pancreatic

[1] Vernon, *Journ. Physiol.*, 27, p. 176, 1901.

[2] Vernon, *ibid.*, 28, p. 470, 1902; 29, p. 331, 1903.

[3] Loew, *Pflüger's Arch.*, 102, p. 95, 1904.

[4] Vernon, *Journ. Physiol.*, 26, p. 405, 1901; 27, p. 269, 1901; 30, p. 350, 1903.

extracts is so unstable that 55 to 75 per cent. of it is destroyed on keeping them for an hour at 37° with ·4 per cent. Na_2CO_3. But extracts which had been kept for some months at room temperature, or for twenty-four hours at 38° with Na_2CO_3, and which had in consequence deteriorated in activity, were more and more stable the greater the deterioration, so that very weak extracts suffered a destruction of only 3 to 7 per cent. of their proteolytic activity when kept for an hour with ·4 per cent. Na_2CO_3. Presumably this was due to the most unstable trypsin groupings first undergoing destruction, whilst the more and more stable ones remained. It was natural to conclude that the extracts contained a number of trypsins of various degrees of stability, but I found[1] that it was impossible to separate stable and unstable trypsins by fractional alcohol precipitation, so it is probable that the tryptic-rennetic molecule contains a number of tryptic side-chains of various degrees of stability, which can be destroyed one at a time independently of one another. The rennet ferment of the pancreatic extracts behaved similarly to the tryptic,[2] so the tryptic-rennetic enzyme must likewise contain a number of rennetic side-chains of various degrees of stability. The erepsin of fresh pancreatic and intestinal extracts also reacted in the same way,[3] so it may be a property of many enzymes, intracellular no less than extracellular, to possess numbers of side-chains of different degrees of stability. It is not a property of all enzymes, however, as the diastatic enzyme of the pancreas showed no such variations of stability.[4] That is to say, fresh and active extracts were no more unstable than old, feebly-acting extracts, or the extracts, when kept in dilute solution in an incubator, showed no more rapid deterioration of ferment activity during the first hour of incubation than in subsequent hours.

Nevertheless it is possible that the pancreas contains more than one tryptic enzyme; for Fermi[5] has shown that the addition of dilute solutions of mercuric chloride, phenol, salicylic and hydrochloric acids to an extract may destroy its power of digesting fibrin, but not that of digesting gelatin. Also

[1] Vernon, *Journ. Physiol.*, 29, p. 302, 1903.
[2] *Ibid.*, 27, p. 195, 1901.
[3] *Ibid.*, 30, p. 330, 1903.
[4] *Ibid.*, 27, p. 197, 1901.
[5] Fermi, *Arch. f. Hygiene*, 10, p. 1, 1890.

Pollak[1] found that if pancreatic extract were acted upon with hydrochloric acid for a few minutes, and then neutralised, it might entirely lose its power of digesting serum proteins, but still retain two-thirds of its original gelatin-digesting power. He supposed that the acid destroyed the trypsin, but left a "glutinase" enzyme comparatively unharmed. But perhaps it is due to the stable tryptic side-chains being more especially suited for the digestion of gelatin, whilst the unstable ones chiefly digest native proteins.

Modern work on immunity, especially the evidence obtained from precipitin formation, has shown us that the serum proteins of one animal differ from those of other animals of different species, and that this difference is the greater the further apart they are genetically. Again, Abderhalden and others have shown that differences of chemical composition exist between the bloods of Carnivora and Herbivora, whilst there is a similarity between the blood of the sheep and ox. It is almost certain, therefore, that the enzymes differ likewise in composition, and that the pepsin of the gastric mucous membrane of man differs slightly from that of the ape, more widely from that of the dog or pig, and more widely still from vegetable pepsins. Correlated with differences of chemical constitution and configuration, we should expect to find differences of action. As far as I am aware, this point has not been tested for proteolytic ferments, but I found[2] that the diastatic ferments of the pancreas of various animals differ considerably. The most convenient method of testing the course of their hydrolytic action upon starch paste is by means of the reaction with dilute iodine solution, and I determined the times required by the digests to reach a definite violet stage, a brown stage, and the achromic point. With an achromic point time of 10 minutes, I found that the extracts of dog's and pig's pancreas took approximately $2\frac{1}{2}$ minutes to digest the starch to the violet stage, and 7 minutes to the brown stage. Extracts of sheep and ox pancreas took 1·1 minute for the violet stage, and 4·3 minutes for the brown stage, whilst extracts of human pancreas took 2·0 minutes for the violet stage and 6·3 minutes for the

[1] Pollak, *Hofmeister's Beitr.*, 6, p. 95, 1906.
[2] Vernon, *Journ. Physiol.*, 28, p. 156, 1902.

brown stage. What these particular colour stages correspond to as regards dextrin formation we do not know, but I found that the rate of formation of maltose was likewise different with the different extracts. The achromic point stage corresponded to the hydrolysis of approximately 58 per cent. of the starch to maltose in all cases, but previous to this point being reached, the pig's pancreas extract formed maltose much more slowly than the sheep and ox pancreas extracts. These extracts, in fact, gave identical results in the progress of their maltose formation.

As might be expected, malt diastase gave the most divergent results of all, both in the formation of maltose, and in the course of the colour reactions. With an achromic point time of 10 minutes, it took only ·5 minute to bring the starch to the violet stage, and 2·5 minutes to bring it to the brown stage.

Of course it is possible, as was suggested to me by Dr Bayliss, that these results are due to the presence of different amounts of independent dextrin-forming and maltose-forming enzymes in the various extracts.[1]

Adsorption.—The precipitability of enzymes by alcohol and salts, and their ready coagulability and destruction on exposure to high temperature, is dependent on their colloidal nature. But they possess other properties typical of colloids, and one of the easiest to demonstrate is the property generally known as *adsorption.* As long ago as 1872 v. Wittich[2] pointed out that fibrin, when placed in a dilute pepsin solution, was able to absorb a good deal of the enzyme, and to fix it so firmly to itself that it was not removed—or at least only in part—by subsequent washing. Other observers have shown that fibrin can similarly absorb trypsin, papain, ptyalin, malt diastase, invertase, and maltase, so probably it can absorb all enzymes. v. Wittich thought that the pepsin chemically combined with the fibrin, but subsequent research has shown that the process is more a physical than a chemical one, and is due to the condensation of a surface layer of enzyme molecules upon the porous fibrin structure. This property of adsorbing dissolved substances is possessed by a large number of bodies in varying degrees. Pepsin is adsorbed in considerable amount by animal

[1] *Cf.* Fränkel and Hamburg, *Hofmeister's Beitr.*, 8, p. 389, 1906.

[2] v. Wittich, *Pflüger's Arch.*, 5, p. 443, 1872.

charcoal, kieselguhr, powdered brick; freshly precipitated barium sulphate, calcium phosphate, magnesium carbonate; lead, copper, and uranium salt precipitates; cholesterin and fatty acids. Dauwe[1] found that coagulated serum and egg proteins, casein, raw and cooked meat, and also gelatin, agar, chondrin, and hæmoglobin energetically adsorbed it, but that clay, quartz sand, glass powder, talc, and magnesium phosphate adsorbed it little if at all. The adsorptive power of a substance depends very largely upon its state of division, for the amount of surface it offers for the molecules of enzyme to condense upon varies inversely as the diameter of the constituent particles. If a particle of a substance 1 mm. in diameter be split up into particles 1μ in diameter, the total surface area is increased a thousandfold.

The physical process of adsorption is often complicated by a certain amount of chemical combination, *i.e.* of the interaction of atoms in accordance with their definite combining weights: but the compounds so formed may be so unstable and easily dissociated that though they are true chemical combinations they may appear to be the result of adsorption processes. In the reaction of a strong acid such as HCl with an equivalent amount of a weak base such as ammonia, one finds that the whole of the acid and base in solution combine together, but as Arrhenius and Madsen[2] point out, this is by no means the case if a weak acid such as boracic acid be allowed to react with the ammonia. In a solution containing equivalent quantities of acid and base, only half of the acid is chemically combined with half the base, and the other halves remain free. If an excess of acid be added, more and more of the base is fixed, but even with five equivalents of acid to one of base, 17 per cent. of the base still remains uncombined. Hence in many cases of absorption of a dissolved substance by a solid it may be impossible to state how far the process is a physical one of adsorption, and how far one of weak chemical combination. In fact, every kind of transitional stage between the two extremes is met with.

The adsorption phenomena of enzymes closely resemble

[1] Dauwe, *Hofmeister's Beitr.*, 6, p. 426, 1905.

[2] Arrhenius and Madsen, see Arrhenius, *Immuno-chemistry*, New York, 1907, p. 174.

those of dyes, and most of our exact information is obtained from observations upon these latter substances.[1] What may be called the "Law of Adsorption"[2] seems to hold equally for both. According to this law we find that if a suitable adsorbent substance is placed in solutions of a dye of progressively diminishing concentration, the amount of dye taken up is relatively larger and larger the more dilute the solution. For instance, Bayliss found that equal amounts of filter paper placed in dilute alcoholic solutions of congo red adsorbed the following proportions of the dye :—

Concentration of Solution.	Proportion of Dye in Solution.	Proportion of Dye in Paper.
	Per cent.	Per cent.
·014	40	60
·012	20	80
·010	9·3	90·7
·008	4	96
·006	1·3	98·7
·004	trace	practically all

In a similar manner pepsin is adsorbed by fibrin to a relatively much greater extent from dilute solutions than from more concentrated solutions (v. Wittich, Dauwe).

A case of adsorption conjoined with true chemical combination has recently been described by Barratt and Edie.[3] Cotton wool was kept with methylene blue solution of known concentration for two to thirty-one days at 45°, and the amount of dye taken up was estimated. The mean results obtained were the following :—

Concentration of Dye at end of Experiment.	Concentration of Dye in Cotton Wool.	Percentage of Dye adsorbed (calculated).
Per cent.	Per cent.	Per cent.
4·061	·76	·29
·292	·72	·25
·0266	·58	·11
·0153	·53	·06
·0028	·49	·02

[1] For full literature and discussion of the theory of staining reactions see G. Mann, *Methods and Theory of Physiological Histology*, Oxford, 1902, pp. 330-370. [2] *Cf.* Bayliss, *Biochem. Journ.*, 1, p. 175, 1906.

[3] Barratt and Edie, *ibid.*, 2, p. 443, 1907.

Barratt and Edie conclude—for reasons stated fully in their paper—that in every case the larger part of the dye, viz. ·47 per cent., was in chemical combination, so that in dilute solution only a very small proportion of it was adsorbed.

Similar cases of adsorption complicated by chemical combination are met with in the case of enzymes. When an enzyme is taken up from solution by a substance upon which it can act, it seems probable—for reasons stated below—that some of it enters into loose chemical combination with the substance. But it is as a rule impossible to prove whether the process is one of adsorption or of chemical union. For instance, it was pointed out by W. A. Osborne that calcium caseinogenate does not pass through a porous clay filter, whilst trypsin does. Bayliss[1] found that if solutions of trypsin and calcium caseinogenate were mixed together and filtered, no trypsin came through; so one might presume that the trypsin was to some extent chemically combined with the caseinogen substrate. However, a mixture of caseinogen and malt diastase likewise yielded no enzyme-containing filtrate, though in this case the caseinogen can only have adsorbed the enzyme.

A curious case of adsorption which has the appearance of chemical combination was noted by Hedin[2] for the intracellular proteases of the spleen. Hedin found that whilst charcoal adsorbed both α- and β-proteases in the same proportion, kieselguhr adsorbed much more of the α-protease. In fact, it was doubtful whether it adsorbed any of the β-protease at all. This specific adsorption must have been a physical phenomenon, as there could scarcely have been a chemical union between the kieselguhr and the α-protease.

The adsorbent power of some substances is very large. For instance, Hedin[3] mixed 80 c.c. of trypsin solution with ·5 gm. of animal charcoal, and found on filtration twenty-four hours later that the whole of the enzyme had been adsorbed. A similar experiment with ·1 gm. of charcoal gave 3·75 units of enzyme in the filtrate, and one with ·05 gm. of charcoal, 25·75 units, whilst the trypsin solution originally contained no less than 750 units of enzyme. The enzyme is somewhat firmly fixed by the

[1] Bayliss, *loc. cit.*, p. 224. [2] Hedin, *Biochem. Journ.*, 2, p. 112, 1907.
[3] Hedin, *ibid.*, 1, p. 483, 1906.

charcoal, for on mixing charcoal containing adsorbed trypsin with dilute caseinogen solution, only 1 to 15 per cent. of the adsorbed enzyme was extracted by the caseinogen and utilised for digestion. This extraction requires time, but at a temperature of 20° it was completed in half an hour or less.[1]

A more typical property than adsorption possessed by enzymes in common with colloids is that of non-diffusibility, but the evidence is very contradictory. v. Wittich stated that pepsin dialysed slightly if the liquid inside and outside the dialysis tube contained ·2 per cent. HCl, but neither Hammarsten or Wolffhügel could confirm the statement. Hoppe-Seyler found that diastase could pass through animal membranes and parchment paper without great difficulty, but Wroblewski could not detect any appreciable dialysis in twenty-four hours. The question has been investigated by Chodschajew[2] with great care. The liquid inside and outside the dialyser contained 1 per cent. of sodium fluoride to prevent sepsis, and the integrity of the dialysis membrane was tested thoroughly after each experiment. Chodschajew found that all the enzymes tested by him, viz. yeast invertase, malt diastase, emulsin, trypsin, and pepsin, dialysed to a very slight extent. As a rule traces of dialysed enzyme could be detected after thirty-six hours, and almost always after eight days' dialysis. Again, Fränkel and Hamburg[3] state, though without giving any experimental details, that malt diastase is diffusible. They find that the diastase is a mixture of starch-liquefying enzymes and of enzymes for converting starch into sugar. The latter enzymes dialyse through parchment paper, but the former do not. Hence their molecules must be of different size.

On the whole, therefore, we are justified in supposing that enzymes can diffuse to an extremely slight extent, but probably a good deal less than proteoses, though more than native proteins. On these grounds it seems probable that enzymes have if anything smaller molecules than native proteins. If the percentage of chlorine in pepsin found by Nencki and Sieber and by Pekelharing be taken as correct, and if it be assumed that

[1] Hedin, *Biochem. Journ.*, 2, p. 81, 1907.

[2] Chodschajew, *Arch. de Physiol.*, 10 (5), p. 241, 1898. Literature here quoted.

[3] Fränkel and Hamburg, *Hofmeister's Beitr.*, 8, p. 389, 1906.

the pepsin molecule contain only a single atom of chlorine, its molecular weight works out at about 7400. This is a somewhat higher figure than the minimum value assigned to some proteins, but the whole evidence is so doubtful that it scarcely warrants discussion.

Upon the optical properties of enzymes no direct determinations of much value have been made. Pekelharing[1] states that his pepsin had a lævo-rotation, for yellow light, of about 50°, or a value similar to that of proteins, but he did not attempt to determine it at all exactly. Much more interesting evidence than this has been obtained by an indirect method. Fischer and Bergell[2] observed that if trypsin were allowed to digest the optically inactive racemic body carbethoxyglycyl-dl-leucin for some days, the solution became dextro-rotatory. It contained an oil which was probably carbethoxyglycyl-d-leucin, and leucin, which was isolated and found to consist for the most part of the lævo-rotatory body. That is to say, the trypsin had hydrolysed one of the two optically active components of the racemic leucin compound more rapidly than the other, and so was itself presumably an optically active substance. Dakin[3] tested the action of the intracellular lipolytic enzyme of the liver upon several of the esters of optically inactive mandelic acid with a similar result. The methyl, ethyl, iso-amyl, and benzyl esters of mandelic acid, $C_6H_5.CH(OH).COOH$, were *partially* hydrolysed by means of the enzyme, and the mandelic acid liberated was strongly dextro-rotatory, whilst the residual ester was correspondingly lævo-rotatory. The free acid never contained more than 38 per cent. of the dextro-rotatory body, over and above that required to neutralise the lævo acid liberated, so even in the earliest stages of hydrolysis a good deal of the lævo ester was acted upon by the enzyme. If the hydrolysis was complete, the mandelic acid formed was optically inactive: *i.e.* it consisted of equal parts of the opposite optical isomers. If the esters are hydrolysed with symmetrical reagents such as acid or alkali, the two optical isomers are split up at the same rate, and the mandelic acid separated at any stage of the

[1] Pekelharing, *Zeit. f. physiol. Chem.*, 35, p. 27, 1902.
[2] Fischer and Bergell, *Ber.*, 36, p. 2592, 1903.
[3] Dakin, *Journ. Physiol.*, 32, p. 199, 1905.

hydrolysis is always inactive. Dakin suggests that in the hydrolysis by lipase the dextro and lævo components of the inactive ester first combine with the enzyme, but in that this is an optically active asymmetric body, they do so at different rates. The compound enzyme *plus* ester is then hydrolysed, but since the compound enzyme *plus* d-ester is not the optical opposite of enzyme *plus* l-ester, the rate of change in the two cases is again different. In the present instance, the enzyme *plus* d-ester is probably formed more rapidly and hydrolysed more rapidly than the other compound.

On the hypothesis that the hydrolysis is influenced by the configuration of the complex enzyme *plus* d- or l-ester molecules, one would naturally expect that the acid liberated from the closely related esters would be of the same sign. As already stated, the dextro-rotatory acid was always formed from the mandelic esters. From the methyl and ethyl esters of phenyl-chloracetic acid and phenyl-bromacetic acid, however, Dakin[1] found that the lævo acid was always liberated first by the action of lipase.

The assumption made by Dakin that the hydrolysis of the substrate is preceded by a combination of the enzyme with it is supported by other evidence. As previously stated, O'Sullivan and Tomson found that invertase required a temperature fully 25° higher to destroy it when cane-sugar was present than when it was absent. This was presumably due to the enzyme forming a loose combination with the sugar, and being protected thereby. Again, certain of the numerous observations made upon the velocity of enzyme action point to a similar conclusion. Enzymes are regarded as catalysts which accelerate, or sometimes retard, the velocity of chemical reactions in the same way as inorganic catalysts. Their action should accordingly conform to the law of mass action, or the amount of chemical change effected by them in a substance in a given time should always bear a constant ratio to the mass of that substance remaining unchanged. Hence the course of the change should be capable of expression as a logarithmic curve, or for unimolecular solutions the expression $\frac{1}{t} \log \frac{1}{1-x}$

[1] Dakin, *Journ. Physiol.*, 32, p. 199, 1905.

(where x is the amount of chemical change induced in the time t) should be constant. This constant, K, is known as the velocity co-efficient, or velocity constant. When this formula is applied to the experimental data obtained with various enzymes, it is found that though it holds for part of the reactions, it does not apply to the initial and final stages. For instance, Adrian Brown [1] found that when invertase was allowed to act upon cane-sugar in solutions of different concentrations, the amount hydrolysed did not bear a constant proportion to the total amount of sugar present, but that a practically *constant weight* was inverted in every case. Thus:

Grams of Cane-Sugar in 100 c.c.	Grams of Cane-Sugar inverted in 60 minutes.	Fraction of Cane-Sugar inverted in 60 minutes.	K.
		Per cent.	
4·89	1·230	25·2	·00210
9·85	1·355	13·8	·00107
19·91	1·355	6·8	·00051
29·96	1·235	4·1	·00031
40·02	1·076	2·7	·00020

On the other hand, in dilute solutions of cane-sugar, when the proportion of sugar to enzyme fell below a certain maximum, Brown found that the amount of sugar hydrolysed was directly proportional to the amount present.

Grams of Cane-Sugar per 100 c.c.	Grams of Cane-Sugar inverted in 60 minutes.	K.
2·0	·308	·00132
1·0	·249	·00219
·5	·129	·00239
·25	·060	·00228

These data show that with 1 per cent. or less of cane-sugar K was constant.

Horace Brown and Glendinning [2] obtained somewhat similar results for the action of malt diastase upon starch, and they lay stress on the fact that in the initial stage of the reaction equal amounts of starch are hydrolysed in equal times, or in other words, the reaction is linear, not logarithmic.

[1] A. Brown, *Journ. Chem. Soc. Trans.*, 1902, p. 373.

[2] H. Brown and Glendinning, *ibid.*, 1902, p. 388.

E. F. Armstrong[1] investigated the action of lactase and maltase, and he found that in addition to an initial linear stage and subsequent logarithmic stage, there is a final stage in which the velocity constant diminishes owing to the influence of the products of action. Bayliss[2] observed the same thing as regards the action of trypsin on caseinogen.

Victor Henri,[3] arguing from his investigations upon the action of invertase, came to the conclusion that part of the enzyme remained free, part combined with the cane-sugar, whilst still another part combined with one of the products of reaction, viz. fructose. Thus he showed that fructose retarded the action of the enzyme, whilst glucose did not. Brown and Glendinning, following the somewhat similar views expressed by Adrian Brown, assume that the hydrolysis is preceded by a combination of the enzyme with the substrate. In the initial stage, when the sugar (or other substrate) is in large excess, the whole of the enzyme is combined with sugar, but the amount of sugar so combined forms only a small fraction of the whole quantity of sugar present. Consequently the enzyme will hydrolyse equal quantities of sugar in equal times. As the concentration of the sugar diminishes, the amount of it at any time in active association with enzyme will be proportional to this concentration, or the rate of change will follow the law of mass action.

Deviations from the law of mass action are brought about, not only by the accumulation of products of action, but by another factor closely related to the retardation so produced, viz. the reversible nature of these reactions. This subject is discussed in detail in the next lecture, together with evidence in favour of a definite union between enzyme and substrate. Still another cause of deviation from the law is found in the gradual destruction of the unstable enzyme which frequently occurs throughout the hydrolysis.[4]

[1] E. F. Armstrong, *Proc. Roy. Soc.*, 73, p. 500, 1904.

[2] Bayliss, *Arch. d. Sci. Biol.*, 11, Suppl., p. 261, 1904.

[3] Henri, *Zeit. f. physik. Chem.*, 39, p. 194, 1901 ; also, *Lois générales de l'Action des Diastases*, Paris, 1903.

[4] For a more detailed account of the velocity of enzyme action, see Moore, *Recent Advances in Physiology*, ed. by L. Hill, London, 1906 ; also, Bayliss, *Sci. Progress*, 1906, p. 281.

LECTURE VII

REVERSIBLE ENZYME ACTION

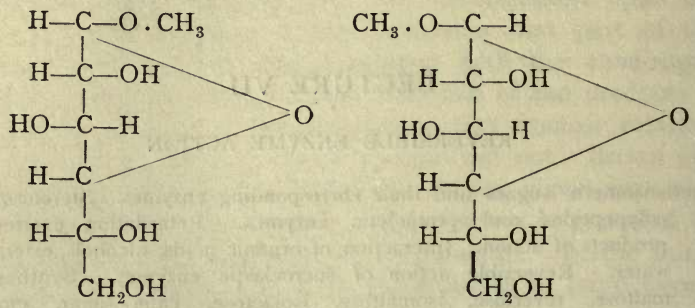
Stereoisomeric sugars and their corresponding enzymes. Stereoisomeric polypeptides and proteolytic enzymes. Retardation exerted by products of action. Interaction of organic acids, alcohols, esters, and water. Reversible action of sucroclastic enzymes. Synthesis of maltose, revertose, isomaltose, isolactose, cane-sugar, emulsin. Synthesis of ethyl butyrate, glycerin triacetate, methyl oleate, mono-olein and triolein by lipolytic enzymes. Action of organic and inorganic catalysts compared. Synthetic action of proteolytic enzymes. Formation of plastein. Energy relations of reacting systems. Transformation of radiant energy of sun into chemical energy by catalytic agents. Synthesis in plants and animals.

WE saw in a previous lecture that each of the three bioses, cane-sugar, maltose, and lactose, is hydrolysed by a special enzyme. Also the trehalose of various fungi is hydrolysed by a specific trehalase enzyme, and the hexatriose sugar raffinose by a specific raffinase. There is evidently, therefore, some close relationship between the structure of a sugar and that of the enzyme which can hydrolyse it. In 1894 Emil Fischer,[1] by his classical researches upon artificial and natural glucosides and their related enzymes, largely extended our knowledge in this field. D-glucose (dextrose) is known to exist in two stereoisomeric forms, and Fischer found that when it is dissolved in methyl alcohol containing hydrochloric acid, two stereoisomeric methyl glucosides are formed, according to the equation :—

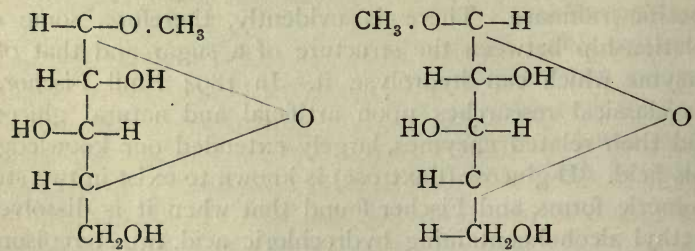
$$C_6H_{12}O_6 + CH_3OH = C_6H_{11}O_6 . CH_3 + H_2O.$$

[1] Fischer, *Ber.*, 27, pp. 2985, 3479, 1894 ; 28, pp. 1429, 1508, 1145, 1895. Summary in *Zeit. f. physiol. Chem.*, 26, p. 60, 1899

According to Fischer these glucosides have the following formulæ, which are identical except as regards the spatial position of the $-O.CH_3$ and the $-H$ radicals attached to the first carbon atom. This difference of configuration is quite sufficient to determine their reaction with enzymes, for Fischer found that one of them, which he called the α-methyl-glucoside,



was readily split up by yeast maltase into glucose and methyl alcohol, whilst the stereoisomeric β-methyl-glucoside was not attacked at all. Emulsin, on the other hand, split up the β-glucoside, but not the α-glucoside. The corresponding α- and β-ethyl-glucosides reacted in the same way with the two enzymes, but none of the glucosides of the pentoses and heptoses prepared by Fischer were attacked by either enzyme. This is a somewhat remarkable fact, for α- and β-methyl-xylosides, for instance, have the following formulæ:—



or differ from the corresponding methyl-glucosides only by the lack of an $H-C-OH$ grouping. Yet this is sufficient to prevent the enzymes from reacting with them, and splitting off methyl alcohol. It would seem, therefore, that when an enzyme acts upon a molecule of a sugar, it comes into contact with it

at a number of different points, perhaps at each carbon atom, and that an alteration in the spatial arrangement of the groupings attached to any one of these carbon atoms may be sufficient to prevent the enzyme from combining or entering into sufficiently close relationship with the sugar molecule to hydrolyse it. On the basis of these results, Fischer suggested that the molecules of enzyme and sugar must be mutually related to one another in the same way as a key is related to the lock which it alone is able to unfasten.

All the natural glucosides hitherto investigated seem to be related to the artificial β-glucosides, as they are hydrolysed by emulsin, and not by maltase. At least this is the case as regards salicin, helicin, æsculin, arbutin, coniferin, and syringin. Saponin, phloridzin, phillyrin, and apiin are not hydrolysed by either enzyme, whilst amygdalin is hydrolysed by both. The action of the two enzymes differs considerably, however, as emulsin splits up amygdalin into benzoic aldehyde, hydrocyanic acid, and glucose, thus:

$$C_{20}H_{27}NO_{11} + 2H_2O = HCN + C_6H_5 . CHO + 2C_6H_{12}O_6,$$

but maltase is only able to split off a single glucose group, and leaves the remaining nitril-glucoside of mandelic acid untouched. Maltose, in that it is hydrolysed by maltase, may be looked upon as glucose-α-glucoside, whilst according to E. F. Armstrong [1] isomaltose, in that it is hydrolysed by emulsin and not by maltase, is presumably the stereoisomeric glucose-β-glucoside.

However, the action of enzymes is not invariably specific. Thus emulsin can hydrolyse β-methyl-glucoside, β-methyl-galactoside, milk sugar and amygdalin, but Fischer does not on this account consider that it contains four distinct enzymes. Again, beer yeast, which ferments mannose, glucose, fructose, and galactose (the d- forms), probably contains only a single zymase. On the other hand, Fischer and other investigators have shown that of the eleven known aldehyde hexoses, only three are acted upon by any of the sucroclastic enzymes, or are fermentable, these being the naturally occurring d-glucose, d-mannose, and d-galactose. The stereoisomeric hexoses pro-

[1] E. F. Armstrong, *Proc. Roy. Soc.*, B. 76, p. 592, 1905.

duced by artificial means cannot be fermented or split up by enzymes. Similarly the naturally occurring d-fructose is the only member of the ketone hexoses which is fermentable or acted on by enzymes.

Similar evidence of correlation between enzyme and substrate has been obtained for proteolytic enzymes and certain polypeptides. Fischer and Abderhalden[1] found that none of the synthetically prepared polypeptides were hydrolysed by pepsin, but that about half of them were attacked by trypsin, and almost all of them by the proteolytic endoenzymes of the liver and other organs. Such of them as were racemic bodies were as a rule hydrolysed asymmetrically, and only a half or a quarter of them split up. On hydrolysis of alanyl-glycin, for instance, only the d-alanyl-glycin isomer was attacked, and the lævo body was untouched, whilst on hydrolysis of leucyl-glycyl-glycin the l- body was attacked, and the d- isomer untouched. Most interesting of all is the case of alanyl-leucin A. This body consists of the four stereoisomers:

d-alanyl-l-leucin,
l-alanyl-d-leucin,
d-alanyl-d-leucin,
l-alanyl-l-leucin,

and only the first of these bodies was hydrolysed by trypsin. Now it is found that the alanin formed by the hydrolysis of native proteins is always the d- form, whilst the leucin is always the l- form. Hence it follows that trypsin can only attack polypeptides of which the amino acid constituents have a similar configuration to those present in native proteins.

Probably enzymes enter into almost as intimate a relationship with certain products of their action as with the substrate they have split up. Tammann[2] showed that the hydrolysis of salicin and amygdalin by emulsin is considerably retarded by the addition of any one of their respective products of action; but Henri found that as regards invertase the retarding effect is practically confined to one of the products of action, viz. fructose,

[1] Fischer and Abderhalden, *Zeit. f. physiol. Chem.*, 46, p. 52, 1905. See also, Abderhalden and Teruuchi, *ibid.*, 47, pp. 159 and 466, 1906; 49, p. 1, 1906; Abderhalden and Hunter, *ibid.*, 48, p. 537, 1906.

[2] Tammann, *ibid.*, 16, p. 291, 1892.

and that glucose has little or no influence. E. F. Armstrong[1] examined the influence of glucose, galactose, and fructose upon the action of each of the four enzymes lactase, emulsin, maltase, and invertase, and tabulated his results as follows:—

Enzyme.	Corresponding Hydrolyte.	Effect of Hexose on rate of change.		
		Glucose.	Galactose.	Fructose.
Lactase	β-galactosides (*i.e.* Milk Sugar, β-alkylgalactosides)	No influence	Retards	No influence
Emulsin	β-glucosides (*i.e.* most natural glucosides): β-galactosides	Retards considerably	Retards slightly	No influence
Maltase	α-glucosides (*i.e.* maltose, α-alkyl-glucosides): α-galactosides	Retards considerably	Retards slightly	No influence
Invertase	Fructosides (*i.e.* Cane-Sugar, Raffinose and Gentianose)	No influence	...	Retards

The substances recorded in the second column of the table are those which, according to Emil Fischer, are alone hydrolysed by the particular enzymes given in the first column. The table shows us that the action of lactase is retarded by only *one* of its products of action, viz. galactose, and that the other product, glucose, does not affect it any more than it affects the action of invertase. We see from the second column that emulsin and maltase can hydrolyse galactosides, but they do not attack them so readily as they do glucosides, just as galactose is fermented less readily than glucose. Galactose differs from glucose merely in a reversal of the $-H$ and $-OH$ radicals attached to the fourth carbon atom, so this difference of spatial arrangement is not sufficient to prevent enzyme action, though it retards it. In a corresponding manner the addition of galactose retards the action of emulsin and of maltase much less than the addition of the more closely correlated glucose.

These results prove without doubt that an enzyme enters into an intimate relationship of some sort with one or more of the products of its activity. It is thereby withdrawn from the sphere of action, and so the hydrolysis undergoes an increasing degree of retardation the further it proceeds. Looked at in another way, we may say that the retardation is produced by a

[1] E. F. Armstrong, *Proc. Roy. Soc.*, 73, p. 516, 1904.

tendency to reverse action. Just as the enzyme enters into intimate relationship with the substrate it is hydrolysing, so it enters into intimate relationship with one or more of the products of its hydrolysis, and in virtue of this intimate relationship tends to bring about a union of the cleavage products. E. F. Armstrong [1] does not believe that the retardation effected by products of action is due to a tendency to reverse action, in that the action of emulsin upon milk sugar is retarded chiefly by glucose, whilst that of lactase upon milk sugar is retarded by galactose. That is to say, the enzyme does not appear to enter into intimate relationship with *both* of the cleavage products formed by its activity. But there is no reason for assuming that such a relationship is essential for the synthesis, as the lactase, for instance, might attach a molecule of galactose to itself, and then combine it with a glucose molecule, without entering into intimate relationship with this molecule. Similarly when lactase hydrolyses lactose, there is no good reason for assuming that it enters into intimate relationship with more than the galactose half of its carbon atoms.

The retardation exerted by products of action, and the tendency to reverse action induced by them, was observed more than forty years ago by Berthelot and Pean de Saint Gilles [2] in their investigations upon the conditions of etherification. They showed that when organic acids and alcohols are allowed to react together, or the corresponding esters and water, the final condition of equilibrium depends only upon the mass of reacting substances, and is not affected by the particular combination in which the substances are brought together. For instance, when ethyl alcohol is heated with an equivalent quantity of acetic acid, the following reaction occurs:—

$$CH_3 - COOH + C_2H_5 - OH = CH_3 - CO - O - C_2H_5 + H_2O;$$

but the ethyl acetate and water formed react with one another to form ethyl alcohol and acetic acid again, and these two reactions proceed simultaneously at different rates until they reach an equilibrium point, *i.e.* a point at which they both

[1] E. F. Armstrong, *Proc. Roy. Soc.*, 73, p. 516, 1904.

[2] Berthelot and Saint Gilles, *Ann. Chem. Phys.* (3), 65, p. 385; 66, p. 5, 1862.

proceed at the same rate. In the case of molecular proportions of these bodies reacting at a temperature of 154°, Menschutkin found that this equilibrium point is such that two-thirds of the acetic acid and alcohol present have combined to form ethyl acetate, and one-third remains uncombined; or, if the ethereal salt and water be kept together at a like temperature, then the reverse action occurs, and a third of the salt is hydrolysed. With increasing amounts of water, the mass action causes the equilibrium point to approach nearer and nearer to the alcohol *plus* acid end of the reaction, as the following data show:—

Molecules of Acetic Acid.	Molecules of Alcohol.	Molecules of Water.	Per cent. of Acid Esterified.
1	1	0	66·5
1	1	3	40·7
1	1	23	11·6
1	2	98	7·3

The equilibrium point varies with different alcohols and acids. Secondary alcohols undergo a smaller amount of esterification than primary alcohols, and tertiary alcohols very much less still. In the presence of catalysts such as acids, the velocity of esterification is greatly increased, and the stronger (*i.e.* the more dissociated) the acid the more it accelerates the velocity of action. Thus Ostwald found that methyl acetate is hydrolysed 300 times more rapidly by hydrochloric acid than by acetic acid. The velocity of hydrolysis of the ester is accelerated by the presence of acids in like proportion, so the equilibrium point is not changed. That is to say, the added acid is purely a catalytic agent, which undergoes no alteration itself, and by its presence neither adds energy to the reacting system, or takes it away.

As far as we know, every enzyme is retarded to a greater or less extent by the accumulation of its products of action, and hence it seems highly probable that no decomposition produced by an enzyme acting in the presence of such products is able to proceed to absolute completion, but reaches an equilibrium point which falls short of it to a greater or less extent. Arguing from the analogy of chemical reactions such as that above

referred to, it seems to follow that every enzyme which can hydrolyse a substance into two or more cleavage products is likewise able to dehydrate them back to the original substance. The synthetic power may be so small as to be undetectable by direct experimental methods, but it must always exist to a greater or less degree.

The first definite proof of the capacity of enzymes to induce reverse or synthetic action was obtained by Croft Hill[1] in 1898. An aqueous extract of dried and pounded yeast containing maltase and other enzymes was allowed to act upon glucose. The dehydration induced was estimated by determinations of the cupric reducing power and the specific rotatory power, and Hill found that when the yeast extract was allowed to act upon 40 per cent. glucose solution, the reducing power gradually diminished, whilst the rotatory power correspondingly increased. On the supposition that the synthetically formed biose consisted of maltose, he calculated that after thirteen days at 30° 7·5 per cent. of maltose was formed; after forty days, 13·5 per cent.; and after sixty-eight days, 15 per cent. Judging from the small amount of dehydration effected in the last twenty-eight days, the equilibrium point must nearly have been reached. Hill endeavoured to show that practically the same equilibrium point was reached, but from the opposite direction, when maltase was allowed to hydrolyse 40 per cent. maltose solution, but further research has shown that the processes of synthesis and hydration occurring in a mixture of yeast extract, maltose, and glucose are not of the simple character at first supposed, and so the complete analogy of this particular type of enzyme action with chemical reactions such as esterification has not yet been established.

Just as the equilibrium point in the alcohol, acid, ester, and water system approaches nearer and nearer to the alcohol *plus* acid end of the reaction, *i.e.* to that of complete hydrolysis, the greater the proportion of water present, so is it in the case of enzyme actions. Or, otherwise expressed, the synthetic power of an enzyme is smaller and smaller the greater the dilution. Hill calculated that in 20 per cent. solution of glucose, maltase would form at most 9·5 per cent. of maltose; in 10 per

[1] Croft Hill, *Journ. Chem. Soc. Trans.*, 1898, p. 634.

cent. solution, at most 5·5 per cent. of maltose; in 4 per cent. solution, 2·0 per cent., and in 2 per cent. solution, 1·0 per cent. Nevertheless, however dilute the solution, there must always be a certain amount of synthetic action.

From the synthetic product formed by the action of maltase upon glucose, Hill prepared a small quantity of maltosazone crystals, and he thought that maltose was the only biose present. Emmerling,[1] who repeated Hill's experiments, concluded that the sugar formed was isomaltose; but Hill,[2] after re-examination of the question, still maintained that maltose was formed, though he found that by far the larger portion of the synthetic product consisted of a hitherto unknown biose, which he named revertose. Whatever be the exact nature of the polymerisation, there can be no doubt that a synthesis of some kind is effected in concentrated glucose solution by the enzymes of yeast extract. The formation of more than one synthetic product is not irreconcilable with the doctrine of reversible enzyme action above expressed, for as Hill points out, one has no right to assume that maltase is the only enzyme concerned in the synthesis. The yeast extract probably contains several different enzymes, each exerting a different action. The important point which he endeavoured to establish by a number of careful experiments, is that the synthetic products formed by the action of yeast enzymes in a concentrated glucose solution are hydrolysed back again to glucose on dilution of the solution. Presumably in this case the maltase which formed the synthetic maltose is likewise responsible for its re-hydration, whilst a "revertase" enzyme acts similarly upon the synthetic revertose. This view is supported by some fermentation experiments. Certain yeasts, such as Saccharomyces Marxianus, ferment glucose but not maltose. Consequently, when added to the product obtained by the action of yeast extract on concentrated glucose solution, it ferments the glucose, but leaves the synthetic bioses unchanged. But if the yeast extract *plus* glucose product be fermented with yeast known to be capable of fermenting maltose as well as glucose, then a small part of the synthetic bioses—presumably the

[1] Emmerling, *Ber.*, 34, pp. 600 and 2206, 1901.
[2] Croft Hill, *Journ. Chem. Soc. Trans.*, 1903, p. 578.

maltose and perhaps higher polymers—is fermented as well as the glucose. However, the larger part of them, which analysis proved to consist almost wholly of revertose, is still unattacked; but if the yeast extract *plus* glucose product is first diluted, whereby its synthetic bioses are hydrolysed back to glucose again, then it can be fermented completely by Saccharomyces Marxianus.

In addition to yeast extract, Hill found that taka-diastase (which contains maltase as well as amylase) has a moderate synthetic action upon concentrated glucose solution, whilst the enzymes of pig's pancreas extract have a slight one. The products of the synthetic action were not identical, but in every case it was found that on dilution they were hydrolysed back again to glucose. The differences of action are presumably due to differences in the nature of the enzymes. The much greater synthesis observed with yeast extract may be due to its containing several different enzymes, each of which exerts a more or less independent synthetic action upon the glucose, and forms a different biose.

Though the existence of reversible enzyme action is generally admitted, the theory of the process above indicated has not passed unchallenged. E. Fischer and E. F. Armstrong[1] found that when a mixture of galactose and glucose was subjected to the action of lactase, reverse action occurred, but the biose formed was not lactose, but the isomeric isolactose. As already stated, Emmerling found that the biose formed by the action of maltase upon glucose is isomaltose. Armstrong confirms this conclusion, and surmises that the revertose described by Hill is identical with isomaltose. Still, he gives but few details in proof of his statement, whilst Hill, on the other hand, prepared pure revertose, and found that in its optical rotation and other properties it differed widely from isomaltose, so for the present we must accept his view as the more probable.

Arguing from this synthetic formation of isolactose and isomaltose rather than of lactose and maltose, Armstrong[2] concludes that "there can be no doubt that the enzyme has a specific influence in promoting the formation of the biose which

[1] E. Fischer and E. F. Armstrong, *Ber.*, 35, p. 3151, 1902.
[2] E. F. Armstrong, *Proc. Roy. Soc.*, B. 76, p. 592, 1905.

it cannot hydrolyse." It is difficult to see the justification for so sweeping a statement, for, as above stated, not only has Hill shown that the synthetic products formed by the action of yeast extract, taka-diastase, and pancreatic diastase are hydrolysed again on dilution of their solutions, but Fischer and Armstrong have themselves observed a similar hydrolysis on diluting the mixture of lactase and sugars containing synthetic isolactose. Again, Visser[1] found that invertase is able to synthesize cane-sugar from a mixture of glucose and fructose, whilst emulsin, which hydrolyses salicin to glucose and saligenin, can dehydrate these substances back to salicin. Still again, Pantanelli[2] found that the invertase of various moulds could synthesize cane-sugar from invert sugar. Hence the weight of evidence, as at present adduced, is considerably in favour of the view that an enzyme can hydrolyse in dilute solution the synthetic products it forms in concentrated solution.

It must be admitted, however, that there are several difficulties which need clearing up. In the first place, it was stated above that the revertose formed synthetically by yeast extract was not fermented by maltase-containing yeasts, hence the extract apparently contained an enzyme which was not present in the living yeast cells. Then Armstrong found that emulsin, when left for two months at 25° with concentrated glucose solution, formed a biose which had the properties of maltose, *i.e.* of a sugar not fermentable by emulsin. However, he does not appear to have determined whether the synthetic biose in his glucose *plus* emulsin mixture underwent hydrolysis on dilution. There can be little doubt that it would have done so.

In previous lectures mention has been made of one or two cases in which abnormal optical isomers were produced by enzyme action. Cathcart[3] found that when α-protease hydrolysed coagulated blood serum proteins the arginin produced was optically inactive, and was not the dextro form which is always obtained on hydrolysis by acids and by other enzymes. Again, Magnus-Levy[4] found that 90 per cent. of the lactic acid

[1] Visser, *Zeit. f. physik. Chem.*, 52, p. 257, 1905.
[2] Pantanelli, *Rendic. d. R. Accad. d. Liucei* [5ª], xvi. 6, p. 419.
[3] Cathcart, *Journ. Physiol.*, 32, p. 299, 1905.
[4] Magnus-Levy, *Hofmeister's Beitr.*, 2, p. 261, 1902.

produced during antiseptic autolyses of various tissues was of the inactive form, whilst in aseptic autolyses over 60 per cent. was of this form. But Mochizuki and Arima[1] found that in the autolyses of bull's testes only the dextro-rotatory acid was formed, and Kikkōji[2] similarly obtained the dextro acid in ox spleen autolyses. These observations, taken in conjunction with those just recorded, seem to indicate that comparatively slight changes in the conditions of action of an enzyme, such as acidity, alkalinity, or the presence of antiseptics, are sufficient in some cases to influence the optical character of the products of hydrolysis. Though in many cases Fischer's simile of lock and key seems to be rigidly applicable, yet it is not so in all, as in the instances adduced on a previous page, and in certain other reactions. Hence it may be that an enzyme which is unable, under most conditions, to attack some particular stereoisomer, is able to do so in the presence of certain abnormal substances such as acids, alkalis, antiseptics, or some related stereoisomers. Possibly maltase, though unable to hydrolyse a pure preparation of isomaltose, can attack it in presence of maltose, glucose, revertose, or some other unknown substance or substances. An explanation of the apparently conflicting results above described will perhaps be arrived at on some such lines as these.

Several other observations have been made upon the synthetic power of various sucroclastic enzymes. Fischer and Armstrong found that kefir lactase could form a bihexose when placed in concentrated glucose solution, whilst emulsin formed one from a mixture of glucose and galactose. The synthetic bodies were not isolated, however. Cremer[3] kept glycogen-free or glycogen-poor yeast juice with 10 per cent. or more of fermentable sugar for twelve to twenty-four hours. The glycogen was tested for by iodine solution, and in four cases some appeared to be formed, whilst in four others the result was negative. A more fully substantiated synthesis is that effected by Emmerling[4] with yeast maltase. On keeping

[1] Mochizuki and Arima, *Zeit. f. physiol. Chem.*, 49, p. 108, 1906.
[2] Kikkōji, *ibid.*, 53, p. 415, 1907.
[3] Cremer, *Ber.*, 32, p. 2062, 1899.
[4] Emmerling, *ibid.*, 34, p. 3810, 1901.

a mixture of 30 gm. of the nitril glucoside of mandelic acid with 18·5 gm. of glucose and 50 c.c. of aqueous yeast extract for three months at 35°, Emmerling found that a small quantity of amygdalin was formed. In this synthesis a molecule of glucose condensed on to a molecule of the nitril glucoside, with separation of a molecule of water.

The synthetic power of lipolytic enzymes has been demonstrated by several independent observers. The hydrolytic action of aqueous extracts of various tissues upon ethyl butyrate and other esters was described in a previous lecture, and under suitable conditions it is found that synthesis of the ester is induced. Kastle and Loevenhart[1] kept 1000 c.c. of pancreatic extract with 1900 c.c. of decinormal butyric acid and 100 c.c. of 95 per cent. alcohol for forty hours at 25°, in presence of thymol, and on distilling the mixture in a slow current of air, they obtained nearly a gramme of ethyl butyrate. A control experiment, carried out with boiled pancreatic extract, gave no ester whatever. Again, Hanriot[2] found that if blood serum were kept with a dilute solution of glycerin and isobutyric acid at 37°, the acidity diminished rapidly owing to the formation of glycerin monobutyrate. A somewhat different type of reaction was investigated by Acree and Hinkins.[3] These observers found that the enzymes present in commercial pancreatin, amylopsin, maltase, taka-diastase, and malt diastase were all able to hydrolyse a dilute solution of triacetylglucose (prepared by heating glucose and acetic anhydride together at 100°). Conversely, on dissolving 1 gm. of glucose and ·25 gm. of acetic acid in 200 c.c. of water, and keeping the mixture with ·5 gm. of pancreatin and toluene at 0°, the acidity diminished in four days by 6·6 per cent. This was presumably due to the formation of glucose acetates.

An ester more closely allied to true fats, viz. glycerin triacetate, has recently been synthesised by enzyme action. A. E. Taylor[4] studied the action of the lipolytic enzyme of

[1] Kastle and Loevenhart, *Amer. Chem. Journ.*, 24, p. 491, 1900.

[2] Hanriot, *Comptes Rendus*, 132, p. 212, 1901.

[3] Acree and Hinkins, *Amer. Chem. Journ.*, 28, p. 370, 1902.

[4] A. E. Taylor, *Journ. Biol. Chem.*, 2, p. 87, 1906. See also, Arrhenius, *Immuno-chemistry*, p. 133, 1907.

the castor-oil seed upon this body, and also upon a mixture of glycerin and acetic acid. In this latter case equilibrium was reached very slowly, but after several months more or less the same end-points were attained as those given by sulphuric acid acting under similar conditions of dilution and temperature (18°).

Concentration.	Per cent. of Hydrolysis by means of		Calculated.
	H_2SO_4	Lipase.	
Per cent.			
·5	88	86	88
1·0	82	79	80·1
2·0	78	70	69·8

The last column of the table gives the values calculated according to Guldberg and Waage's law of mass action, on the assumption that the first value (88 per cent.) is correct. In that both the lipase and the acid act only as catalytic agents, the end-points ought to be identical, and to agree with the calculated values. The cause of the discrepancies was not ascertained, but they seem too large to be attributable to experimental error.

Bodenstein and Dietz[1] studied the hydrolytic action of pancreatic lipase on amyl butyrate, and its synthetic action upon butyric acid and isoamyl alcohol. They kept a preparation of alcohol- and ether-washed pig's pancreas with mixtures of ester and amyl alcohol containing 6·5 to 8 per cent. of water,

Per cent. of Water present.	Free Acid remaining in mixture of Acid + Alcohol.	Free Acid liberated from mixture of Ester + Alcohol.	Calculated Equilibrium Point.
6·5	10·58	10·00	10·16
6·5	28·75	27·67	27·91
8	48·51	44·40	45·90
8	33·58	32·85	32·96

[1] Bodenstein and Dietz, *Zeit. f. Electrochem.*, 12, p. 605, 1906; Dietz, *Zeit. f. physiol. Chem.*, 52, p. 279, 1907.

and found that the same equilibrium points were gradually approached as when the ferment was kept with equally concentrated solutions of butyric acid and alcohol. The data in the table show the acidity finally attained in several pairs of experiments, and the real equilibrium points calculated by extrapolation.

Dietz also investigated the action of inorganic catalysts on the synthesis and hydrolysis of the ester, and the data in the table show the equilibrium points attained when 8 per cent. of water was present. It will be seen that they are practically identical for the two acids tried, but that they differ consider-

	Ester formed from Acid+Alcohol.	Acid formed from Ester.
	Per cent.	Per cent.
Equilibrium point with Hydrochloric Acid . .	85·55	14·46
„ „ Picric Acid . . .	85·49	14·50
„ „ Pancreatic Lipase . .	about 75·0	about 25·0

ably from those induced by the lipase. Dietz thinks that this difference may be due to the enzyme adsorbing some of the reacting substances, and so undergoing a physico-chemical change which caused it to induce a different equilibrium point.

A very complete series of experiments upon the synthesis of true fats has been carried out by Pottevin.[1] A dried preparation of alcohol- and ether-washed pig's pancreas was used as the catalytic agent, and in the first group of experiments Pottevin kept 50 gm. of oleic acid and 5·7 gm. of methyl alcohol with 2·5 gm. of the pancreas powder at a temperature of 33°. The acidity of the mixtures was measured at intervals, and from the data in the table we see that in absence of water 85 per cent. of the oleic acid was esterified. In the presence of increasing quantities of water the esterification was smaller and smaller, as we should expect from the law of mass action. At each dilution the equilibrium point was nearly reached in eight days, and completely so in ten or fourteen days.

[1] Pottevin, *Ann. de l'Inst. Pasteur*, 20, p. 901, 1906.

Time.	Grammes of Water present.					
	0	7	14	35	70	140
12 hours .	15	12	10	...	...	...
36 „ .	40	35	32	23	17	...
60 „ .	60	52	45	35	27	...
4 days .	75	68	60	46	32	9
6 „ .	83	76	70	53	...	...
8 „ .	84	78	73	54	38	11
10 „ .	85	79	74	...	...	...
14 „ .	85	80	74	55	40	13

As likewise follows from the law of mass action, the equilibrium point was not affected by the quantity of enzyme added: but as can be seen from the data adduced, the velocity of esterification was greatly accelerated by increasing the enzyme.

Per cent. of Pancreas Powder added.	Per cent. of Oleic Acid esterified in		
	1 day.	8 days.	20 days.
1	8	56	84
2	12	66	82
5	27	66	84
10	43	74	85

The other primary alcohols, viz. ethyl, propyl, isopropyl, butyl, and isoamyl alcohols, showed practically the same degree of esterification as methyl alcohol, and so did secondary butyl alcohol, but isobutyl alcohol and tertiary butyl alcohol hardly reacted at all.

In the second group of experiments Pottevin describes the synthesis of both mono-olein and triolein. To obtain the former body, 100 gm. of glycerin extract of pancreas were kept at 35° with 100 gm. of oleic acid. After eight days the acidity of the mixture had diminished by a third, and 27 gm. of mono-olein were isolated from it. On dissolving mono-olein in fifteen times its weight of oleic acid, and keeping the mixture at 35° with 1 per cent. of its weight of pancreas powder, the acidity gradually diminished, and after about a month reached a constant value. This was due to the formation of triolein, for 14·5 gm.

of this fat were isolated. The considerable synthetic effect observed by Pottevin was due to the possibility of carrying out the esterification in the presence of very little water. If no water whatever were present, however, the esterification of the oleic acid hardly occurred at all, as the following data show :—

40 GM. OF OLEIC ACID + 3 GM. OF PANCREAS POWDER, KEPT FOR 20 DAYS AT 33°, WITH :—

130 gm.	Anhydrous	Glycerin	+	0 gm.	Water	=	3 per cent.	of Acid esterified.
120	„	„		10	„	=	77 „	„
110	„	„		20	„	=	64 „	„
100	„	„		30	„	=	51 „	„
64	„	„		66	„	=	20 „	„
28	„	„		102	„	=	5 „	„
8	„	„		122	„	=	0 „	„

The water must therefore play a definite part of some sort in the interaction of the glycerin and oleic acid.

In that mono-olein and triolein are readily hydrolysed by pancreas powder when in dilute solution, it follows that the reaction induced by the lipolytic enzyme is a strictly reversible one.

In the light of these experiments, it appears that fats and fatty acids afford much better material for the study of reversible enzyme action than carbohydrates. The reactions which occur are simpler, in that fats do not contain asymmetric carbon atoms, and so do not form stereoisomers. Also, the ease with which concentrated solutions can be employed is an important factor. In respect of both these conditions proteins are much less suitable for study even than the carbohydrates. It is not surprising, therefore, that very little positive evidence has as yet been obtained of the synthesis of true proteins by enzyme action. In that proteins are built up of numbers of amino acid molecules linked on to one another with the formation of $-CO-NH-$ groupings, it seems probable that if enzymes are found to be capable of binding two amino acid molecules together with the formation of such a grouping, there is no reason why they should not be able to unite three or four, or in fact almost any number of amino acid molecules, until physical reasons such as small solubility or colloidal nature of the product checked the synthesis. Taylor[1] attempted to form the synthetic

[1] Taylor, " University of California Publications," *Pathology*, 1, p. 65.

peptides of Fischer by the action of trypsin upon the appropriate amino acids, whilst Abderhalden and Rona[1] examined the action of the enzymes of liver juice upon them with a similar object. In both instances the results were negative, but Taylor[2] has recently described the synthesis of a protamine by the action of a proteolytic enzyme obtained from the liver of the soft-shelled California clam. Four hundred grammes of protamine sulphate prepared from the *Roccus lineatus* were dissolved in 15 litres of water, and digested with the enzyme in alkaline solution. The sulphuric acid was removed by precipitation with barium chloride, and the filtrate concentrated to about 5 litres. This solution of free amino acids and their carbonates, the products of cleavage of the protamine, was kept at room temperature with glycerin extract of the clam livers and toluol for five months. The mixture kept quite sterile, and after the addition of sulphuric acid to it, and precipitation with alcohol, a final pure product weighing 1·8 gm. was obtained, which in its solubility, precipitability by alcohol and salts, its digestibility by trypsin and resistance to pepsin, and in its percentage composition, was practically identical with the protamine sulphate originally hydrolysed. Some of the solution of protamine cleavage products, when tested directly without previous enzyme treatment, yielded no protamine at all, so the product isolated in the chief experiment must have been synthesised by the liver enzyme.

Indications of reversible action have been obtained by Bayliss[3] in the case of trypsin. A 40 per cent. solution of the products of hydrolysis of caseinogen was exposed to the action of this enzyme, and Bayliss found that the electrical conductivity of the mixture diminished some 27 per cent. in the course of four days, and then returned again to its original value. In the hydrolysis of caseinogen by tryptic action, the conductivity rapidly increases owing to the splitting up of large molecules into smaller ones, and hence a diminution of conductivity seems to imply the reverse process.

The reversible action of trypsin is also suggested by the

[1] Abderhalden and Rona, *Zeit. f. physiol. Chem.*, 49, p. 31, 1906.
[2] Taylor, *Journ. Biol. Chem.*, 3, p. 87, 1907.
[3] Bayliss, *Arch. d. Sci. Biol.*, 11, Suppl., p. 261, 1904.

retardation exerted by the products of action. I found[1] that the addition of 1 per cent. or less of proteoses and peptones to a solution of trypsin in ·4 per cent. Na_2CO_3 had very little influence upon its fibrin-digesting powers, but with 2 per cent. of these products its activity was reduced by 12 to 21 per cent.

	Relative Tryptic Value in presence of		
	·5 per cent.	1 per cent.	2 per cent.
Proto-proteose	100	86	82
Deutero-proteose	103	98	79
Witte's Peptone	104	98	88
Antipeptone (Kühne) . . .	100	97	84
Witte's Peptone, 81% hydrolysed .	105	100	81
Glycin	111	94	...
Leucin	97	103	...

Witte's peptone which had been digested with trypsin and intestinal erepsin until 81 per cent. of it had been hydrolysed into products no longer yielding the biuret test, exerted no more retardation than less hydrolysed products, whilst individual amino acids such as glycin and leucin, if in 1 per cent. strength, exerted little or no retardation. In all these experiments the tryptic power of the trypsin in absence of any decomposition products was taken as 100.

More detailed experiments upon the retardation exerted by protein decomposition products have recently been made by Abderhalden and Gigon.[2] Yeast press juice was allowed to act upon the polypeptide glycyl-l-tyrosin, and it was found that all of the optically active amino acids which are formed on the hydrolysis of proteins, so far as they were investigated, greatly retarded the hydrolysis. In most experiments 1 c.c. of yeast juice was allowed to act upon ·1 gm. of glycyl-l-tyrosin, and ·1 gm. of the amino acid was added. The acids tested were l-leucin, d-alanin, l-serin, l-phenylalanin, d-glutaminic acid, d-tryptophan, and l-tyrosin. The corresponding antipodes to these bodies, so far as they were tested, had little or no inhibitory influence upon the hydrolysis, whilst the racemic bodies had an intermediate

[1] Vernon, *Journ. Physiol.*, 31, p. 346, 1904.

[2] Abderhalden and Gigon, *Zeit. f. physiol. Chem.*, 53, p. 251, 1907.

effect, as might be expected. Glycin, which is not an optically active amino acid, had little or no influence on the course of action, and so presumably it does not enter into relationship with the enzyme. But the fact that each and all of the optically active amino acids present in native proteins retarded the hydrolysis is extremely suggestive. When we talk of the configuration of an enzyme being adapted to that of its related substrate, we do not necessarily imply that there is any close similarity of chemical structure between their molecules, or parts of their molecules. But this work of Abderhalden and Gigon suggests firstly that the proteolytic enzyme of yeast juice is a protein-like body containing each and all of the constituent amino acid groups normally present in proteins, and secondly that the correlation between enzyme and related substrate is due to their molecules containing one or more groupings of similar or identical chemical structure. That is to say, it suggests that the active portion of an amylolytic enzyme which comes into direct relationship with the carbohydrate it is hydrolysing, has itself a carbohydrate-like structure: that the active portion of a lipolytic enzyme has an ester-like structure, and so on. However, it must be remembered that these views are suggestions only, and very far from being proved.

Synthetic powers have been attributed to the rennin or pepsin of gastric juice by several observers. In 1886 Danilewsky[1] noted that if rennet ferment were allowed to act upon a solution of proteoses and peptones, it gave rise to a flocculent precipitate. Sawjalow[2] named this product plastein, and he and others have investigated its properties, and consider it to be a dehydration product of proteoses. The evidence concerning it is extremely contradictory, for Sawjalow first found that a plastein could be prepared from hetero-proteose and from proto-proteose, and to a less extent from deutero-proteoses. Lawrow and Salaskin[3] obtained it from all of the proteose fractions separated from Witte's peptone by Pick's method. Kurajeff[4] obtained it by the action of gastric juice on secondary

[1] Danilewsky, cited by Kurajeff, *Hofmeister's Beitr.*, 1, p. 121, 1901.
[2] Sawjalow, *Pflüger's Arch.*, 85, p. 171, 1901.
[3] Lawrow and Salaskin, *Zeit. f. physiol. Chem.*, 36, p. 277, 1902.
[4] Kurajeff, *Hofmeister's Beitr.*, 1, p. 121, 1901 ; 2, p. 411, 1902.

proteoses, but not on primary proteoses, whilst he found that papain gave a plastein precipitate with primary proteoses, but not with secondary proteoses. Wait[1] fractionated the proteoses of Witte's peptone with alcohol, and found that the secondary proteoses gave a plastein precipitate with gastric juice, whilst the primary ones did not. Bayer[2] found that no single proteose gave any plastein, though a mixture of them did, and Sawjalow[3] confirms this conclusion, and says that a mixture of all the primary and secondary proteoses is necessary. He attributes the opposite results obtained by himself and other observers to impurities in the proteose preparations.

A plastein precipitate is obtainable not only from Witte's peptone, but probably from every protein. Sawjalow prepared it from egg albumin and globulin, serum albumin and globulin, edestin, myosin, and casein, and he found that its percentage composition was almost constant. It contained on an average 55·3 per cent. of carbon, 7·4 per cent. of hydrogen, 15·0 per cent. of nitrogen, 1·16 per cent. of sulphur, and 21·2 per cent. of oxygen, or had the composition of proteins. Wait and Lawrow obtained similar figures for their analyses, but Kurajeff and Rosenfeld[4] found their plastein preparations to contain about 59 per cent. of carbon. Sawjalow admits that the composition is only constant if the plastein be obtained under certain conditions, for he himself found that when casein was digested two days it gave a plastein containing 55·7 per cent. of carbon, and when digested four days, one containing 57·1 to 59·0 per cent. of carbon. Bayer obtained a plastein containing 38·4 per cent. of carbon and 8·0 per cent. of nitrogen, but his product is so different from those obtained by all other investigators that it must be an entirely distinct substance. Thus it did not give the biuret, xantho-proteic, or lead sulphide reactions.

Plastein is an acid body, insoluble in water, but it dissolves in alkalis to form a soluble alkali salt, and from the amount of

[1] Wait, Diss. in Russian, cited by Sawjalow, *Zeit. f. physiol. Chem.*, 54, p. 119, 1907.

[2] Bayer, *Hofmeister's Beitr.*, 4, p. 554, 1904.

[3] Sawjalow, *Zeit. f. physiol. Chem.*, 54, p. 119, 1904.

[4] Rosenfeld, cited by Sawjalow, *loc. cit.*

alkali necessary to dissolve it, Sawjalow calculated its molecular weight to be about 6000. This is at least double the molecular weight of proteoses. Sawjalow thinks that plastein is formed by the reverse action of the pepsin of the gastric juice, as the reaction goes on well only in concentrated proteose solutions (30 to 40 per cent. being best), whilst in dilute solutions the plastein dissolves up again with formation of primary and secondary proteoses and peptones.

A synthesis of similar character to that occurring in the condensation of amino acids to polypeptides is found in the dehydration of benzoic acid and glycin to hippuric acid. This body is hydrolysed by the endoenzymes of minced kidney substance, but Berninzone[1] states that if the kidney tissue is allowed to act upon a mixture of glycin and benzoic acid in presence of NaF, it is able to synthesise small quantities of hippuric acid. Abelous and Ribaut[2] confirm this result, but the amount of synthetic product obtained by them was extremely small. For instance, a mixture of 425 gm. of chopped horse's kidney, kept with 500 c.c. of horse's blood, 1·5 gm. of glycin, 3 c.c. of benzyl alcohol, and 2 per cent. of NaF for forty-two hours at 42° in a stream of air, yielded only ·11 gm. of hippuric acid.

Sufficient evidence has been adduced to prove that individual members of all classes of enzymes are able, under suitable conditions, to act synthetically, and hence we may assume with a considerable degree of probability that all enzymes, endoenzymes no less than exoenzymes, can act in this way. It seems highly probable, also, that enzymes synthesise the substances which they hydrolyse, or that they are merely catalytic agents which accelerate the velocity of two chemical reactions, occurring in opposite directions, so as to hasten the approach to an equilibrium point. They act in the same way as inorganic catalysts, therefore, except than in virtue of their asymmetric character they may act at unequal rates upon opposite optical isomers. The catalytic agent of itself neither adds energy to a reacting system or takes it away, but the passage towards the equilibrium point, from whichever end of

[1] Berninzone, *Att. d. Soc. ligust. d. Scienc. Nat.*, 11, 1900.
[2] Abelous and Ribaut, *C. R. Soc. Biol.*, 52, p. 543.

the reaction it occurs, is always accompanied by a liberation of energy of some kind. It is this energy which forces on the reaction towards the equilibrium point. Definite knowledge of the energy relations concerned in the reversible actions above described is wanting, but we know that the heat evolved or absorbed during their progress is so small as to be practically inappreciable. It cannot be measured directly, but some idea of its magnitude can be obtained by comparing the heat of combustion of a substance with that of its hydrolytic products. For instance, it is found that in the hydrolysis of cane-sugar to glucose and fructose only 31 rational calories are evolved, or ·23 per cent. on the heat of combustion of this sugar to water and CO_2.[1]

$C_{12}H_{22}O_{11}$ + H_2O = $C_6H_{12}O_6$ + $C_6H_{12}O_6$ + 31K
Cane-Sugar. Glucose. Fructose.
13527K = 6737K + 6759K + 31K

v. Lengyel[2] digested egg albumin with pepsin for two to ten days, and found that the heat of combustion of a definite quantity of the mixture after digestion was 3736 calories on an average, as compared with a value of 3739 calories before digestion. Hari[3] endeavoured to determine the energy loss in tryptic digestion, but he could not find that there was any liberation of energy whatever as the result of hydrolytic processes. That such hydrolysis does occur is proved by the analyses of Möhlenfeld,[4] Kossel,[5] and Danilewsky. Hari found that the digested protein showed a greater and greater increase in weight the more prolonged the digestion, and after four to sixty-five days' tryptic digestion, the increase amounted to 1·1 to 7·2 per cent. In the case of fat hydrolysis, there is likewise no appreciable liberation of energy. A gramme molecule of ethyl butyrate was found to give 851·3 calories on combustion, whilst gramme molecules of ethyl alcohol and of butyric acid together gave 850·1 calories. Again, a gramme molecule of stearin gave 8393 calories,

[1] Quoted from article by B. Moore in Hill's *Recent Advances in Physiology*, p. 40, London, 1906.

[2] v. Lengyel, *Pflüger's Arch.*, 115, p. 7, 1906.

[3] Hari, *ibid.*, 115, p. 52, 1906.

[4] Möhlenfeld, *ibid.*, 5, p. 390, 1872.

[5] Kossel, *Zeit. f. physiol. Chem.*, 1879.

whilst 3 molecules of stearin and 1 of glycerin gave 8413 calories.[1]

It follows that though enzyme action is able to account for a certain amount of synthesis under suitable conditions, it is unable, as at present understood, to explain the storing up of energy. Such storage of energy is constantly taking place in living cells, and is most strikingly instanced in the formation of starch from carbonic acid and water by chlorophyll-containing plant cells. The energy stored up in the starch grains is in this case derived from the sun's rays, or radiant energy from an external source is by some mechanism unknown to us seized upon and transformed into chemical energy. From ignorance of the true explanation, such a transformation of energy is often attributed to some "vital" property of the cell, though similar reactions apart from living cells have long been known to chemists. For instance, Berthelot found that acetylene was produced by passing electric sparks between carbon points in an atmosphere of hydrogen. In the presence of nitrogen, this acetylene formed hydrocyanic acid. Sir Benjamin Brodie found that under the influence of electric discharges carbon monoxide and hydrogen unite to form methane and water. Again, hydrogen and iodine combine together at a red heat to form hydriodic acid, with considerable absorption of energy. It may be objected that these syntheses only occur at very high temperatures, and can have no bearing on any possible changes occurring in living cells; but for aught we know to the contrary, some catalysts may exist in the cells which are able to effect similar energy tranformations at low temperatures. Bach[2] stated that if carbon dioxide were passed through a 1·5 per cent. solution of uranium acetate exposed to sunlight, a precipitate of uranium peroxide and lower oxides was thrown down, whilst formaldehyde and hydrogen peroxide were formed in solution. The uranium oxides presumably acted as a catalytic agent, and enabled the radiant energy of the sun to be transformed into the chemical potential energy stored up in the formaldehyde. A repetition of this experiment by Euler[3] confirmed the synthesis, but the

[1] Quoted from Leathes, *Problems in Animal Metabolism*, p. 76, London, 1906.

[2] Bach, *Comptes Rendus*, 116, p. 1145, 1893.

[3] Euler, *Ber.*, 37, p. 3415, 1904.

product formed was found by him to be formic acid and not formaldehyde. Usher and Priestley[1] likewise found formic acid, but they endeavoured to prove that formaldehyde is an intermediate product. They found that the amount of decomposition in three weeks of bright weather is extremely small, but that the reaction takes place more rapidly if the CO_2 is under considerable pressure. A 2 per cent. solution of uranium sulphate acted in the same way as the acetate, so the formic acid was not derived from the acetic acid of the salt.

Bach thought that the chemical change primarily effected by the sunlight and uranium salt was as follows:—

$$CO_2 + 3H_2O = H.CHO + 2H_2O_2.$$

The hydrogen peroxide, if actually formed in this way in the green plant, would be at once split up into water and free oxygen by the universally present catalase enzyme. Usher and Priestley state that the catalase is strictly localised in the chloroplasts of the green leaf, and though this is improbable, we may conclude that it is more concentrated in these structures than in the rest of the leaf. Formaldehyde is an extremely poisonous body, so if produced in the green plant it doubtless undergoes a further transformation almost immediately. Loew[2] has shown that certain chemical substances such as metallic oxides and sulphites are able to condense formaldehyde to various carbohydrates such as formose, α-acrose and methylenitan. Hence it is possible that a similar condensation is effected in the plant cell by the action of an enzyme. If such an enzyme exists, it seems to be a very unstable body, intimately bound up with the vitality of the cell, for Usher and Priestley found that exposure of *Elodea* to chloroform vapour for two hours destroyed its power of condensing formaldehyde.

Usher and Priestley showed that if green sprigs of *Elodea* were killed by dipping in boiling water for thirty seconds, and were then placed in water saturated with CO_2 and exposed to sunlight, the green colour of the leaves disappeared in a few hours, and the bleached leaves contained formaldehyde. They explain

[1] Usher and Priestley, *Proc. Roy. Soc.*, B. 77, p. 369, 1906; B. 78, p. 368, 1906.

[2] Loew, *Ber.*, 21, p. 271, 1888.

the course of events by supposing that hydrogen peroxide and formaldehyde are at first produced from the CO_2 and water in the normal way, but that the peroxide, instead of being decomposed by catalase as in living plants, oxidises the chlorophyll to a colourless substance. The reaction thereby comes to an end, for the chlorophyll is supposed to be the catalytic agent which induces the reaction.

Some of Usher and Priestley's experiments have been repeated by Ewart,[1] and he finds that chlorophyll forms an aldehyde when exposed to light, but he thinks that it is merely a decomposition product of the chlorophyll, as it is formed even if no carbon dioxide be present. He points out that chlorophyll is bleached by sunlight in presence of air or oxygen containing no CO_2, and he says that there is no satisfactory proof of the formation of hydrogen peroxide or of free oxygen by the agency of chlorophyll, except inside the living cell. Hence we must admit that for the present the synthesis of formaldehyde or formic acid from carbon dioxide and water by an organic catalyst has not been established.

Anabolic processes accompanied by a storage of chemical energy are not limited to chlorophyll-containing cells, but probably occur in every living cell. In the absence of chlorophyll, the protoplasm must derive the energy necessary for the synthesis from some source other than the sun's rays. Usually it comes from the heat produced by the oxidation processes occurring in the cell, or the energy set free by one reaction is used to assist another reaction. Moore[2] points out that it is this "linkage of one reaction with another, and the using of the free energy of one to run another, which specially characterises the cell and differentiates it from the enzyme," and that in the process "a set of manifestations peculiar to life appear, which cannot be reproduced elsewhere than in living cells." Somewhat inconsistently, Moore himself adduces an example of linked reactions from inorganic chemistry, viz. that of hydrogen peroxide upon certain metallic oxides, as those of silver and gold. Hydrogen peroxide spontaneously undergoes a slow conversion into water and oxygen, with evolution of

[1] A. J. Ewart, *Proc. Roy. Soc.*, B. 80, p. 30, 1908.
[2] Moore, *loc. cit.*, pp. 49 and 135 *et seq.*

energy; but in the presence of one of these oxides the velocity of reaction is enormously increased, though much of the energy liberated is absorbed in order to induce another reaction, viz. the reduction of the oxide to the free metal. As Moore points out, "the induced reaction runs the inducing reaction backwards away from its equilibrium point by means of the energy which would otherwise be set free."

In living cells it is true that at present we know little or nothing of the linkage of reactions, but their elucidation may be only a question of time. In the plant cell we see that a catalyst, perhaps chlorophyll, can transform solar energy into chemical potential energy, and that possibly an enzyme may then condense the synthetic formaldehyde into an actual carbohydrate. In the animal cell, most of the syntheses such as the formation of glycogen from glucose, of protein from amino acids, and of neutral fat from glycerin and fatty acids, are accompanied by an extremely small absorption of energy, and, as Moore suggests, the variations of osmotic energy with changes in concentration may account for the energy required, and an enzyme which adds no energy to the reacting system may effect the conversion. But other chemical changes occur in animal cells, such as the transformation of carbohydrates into fats, in which there is a very large absorption of energy. This energy is presumably derived from the heat of combustion of some of the food material stored up in the cell, by the same kind of mechanism as that which in the plant converts solar energy into chemical energy.

Hence it is of paramount importance for us to discover catalytic agents which can effect the transformation of heat energy into chemical energy, and investigate their action. As far as I am aware, no instances of such energy transformation are known to occur at temperatures compatible with the life of a cell, but they are not unknown at higher temperatures. For instance, in the vaporisation of ammonium chloride the vaporised salt, under ordinary circumstances, is dissociated into ammonia and chlorine with absorption of heat. On cooling, the gases combine together to form ammonium chloride, with the evolution of a large amount of heat. How much of this heat is due to chemical combination, and how much to con-

densation from the gaseous to the solid state, we do not know. H. B. Baker[1] has shown that if the ammonium chloride be perfectly dry, it does not dissociate at all on vaporisation, and so it seems to follow that the traces of water usually present act as a catalytic agent, and bring about a transformation of heat energy into chemical energy.

[1] Baker, *Journ. Chem. Soc. Trans.*, 1894 and 1898, p. 422.

LECTURE VIII

ENDOENZYMES AND PROTOPLASM

Comparison of living and dead tissues. Disintegration effected by chloroform and by lactic acid saline. Autodigestion of intact and of minced tissues. Comparison of enzymes with agglutinins and lysins. Zymoids. Antiferments. Constitution of biogens. Action of antiseptics on living organisms and on enzymes. Influence of temperature on enzymes, on metabolism of organisms, on heart beat and on rate of propagation of nervous impulse. Optimum and maximum temperatures.

WE have seen in the preceding lectures that from dead disintegrated tissues endoenzymes can be extracted which are capable of inducing most of the katabolic changes known to occur in these tissues whilst still living. We have seen also that enzymes possess synthetic powers, and so may be responsible for some at least of the anabolic changes which occur in living tissues. We therefore know a good deal about many of the individual groups which, bound up together and acting in harmony with one another, constitute living substance. A far more difficult problem is to discover the manner in which these constituent groups are united to one another, and can exert their activity whenever it is required, independent of what the other constituent groups may be doing. A few years ago we were in total ignorance of this matter, but thanks to the labours of Ehrlich and others, we are now making some progress towards its solution. We regard the unit of living substance, the biogen, as consisting of a nucleus with which numerous side-chains are connected. These side-chains are in most cases of a protein-like nature, and owe their different capacities partly to differences of chemical composition, but

perhaps more especially to differences of structure and configuration. At present we know very little about the chemical differences of these side-chain groups. Still more ignorant are we as to the nature of the linkages which bind them to the biogen nucleus. Any violent chemical or physical treatment of living matter made with a view to determining its chemical constitution, inevitably kills it, and so the evidence yielded by such analysis pertains only to the dead and disintegrating tissue, and does not necessarily hold in any degree whatever for living protoplasm. In fact it is generally held that there exists a fundamental difference between living and dead substance, but extended research may prove that this view is not justifiable, and that the difference is only one of degree, not of kind. Hence a study of the properties of dead and dying tissues may prove of great value in assisting us to understand and account for the seemingly inexplicable properties of living tissues.

A convenient method of studying the properties of dead and dying tissues is that adopted by the writer,[1] and already referred to in a previous lecture. It consists in taking a fresh and still living organ, such as the kidney or heart of a mammal, and perfusing it with saline or some other liquid for several days. The products of disintegration of the tissue, at the time of death and subsequently, are carried away in the perfusion liquid, and can be analysed qualitatively and quantitatively. A kidney perfused continuously in this way at room temperature generally shows very little disintegration for several days if putrefaction be prevented. Throughout the whole of this time, however, the side-chains remain bound to the tissue framework by very weak bonds, which are readily snapped by almost any change in the conditions of perfusion. A temporary stoppage of the flow of perfusion liquid or a change in its salinity, may cause an immediate disintegration of the tissues, whereby considerable quantities of proteins and other substances are washed out of the organ. These other substances seem to consist entirely of protein decomposition products, and contain no appreciable quantity of fat or carbohydrate. As much as 74 per cent. of the total amount of protein present in the

[1] Vernon, *Zeit. f. allgem. Physiol.*, 6, p. 393, 1907.

kidney tissues may be removed by perfusion: hence the statement made above that the side-chains are of a protein-like nature. The products removed by perfusion probably include most of the endoenzymes. Thus the only one investigated by me, endoerepsin, might be almost completely removed by a week's perfusion. The most effective method of all for breaking the link between side-chain and tissue framework is to perfuse with saline saturated with ether or chloroform. In one experiment, a kidney was perfused with oxygenated saline for the first two hours, and then saline saturated with ether was substituted. The disruption was so considerable that 19 per cent. of the total nitrogen and 16 per cent. of the total erepsin in the kidney was washed out in the first three hours of etherisation. In another experiment, a kidney was perfused for 150 hours with 2 per cent. sodium fluoride, and as can be seen from the data in the table, the amounts of erepsin and of protein washed out during this time were extremely small. Ringer's solution saturated with chloro-

Time of Perfusion of Kidney.	Perfusion Liquid.	Substances washed out per hour per 1 kg. of Kidney.			
		Erepsin.	Protein.	Nitrogen ×6·25.	Non-protein Nitrogen.
Hours.			gm.	gm.	gm.
3 to 12	2% Sodium Fluoride .	·003	·022	·123	·108
12 „ 48	„ „ .	·005	·013		
48 „ 95	„ „ .	·064	·029	·086	·057
95 „ 150	„ „ .	·245	·070	·106	·036
150 „ 151	Ringer's Solution saturated with Chloroform	38·46	19·62	19·98	·034
151 „ 156	„ „	1·36	1·83	1·82	
156 „ 168	„ „	·28	·40	·43	

form was then substituted, and within the next hour 12 per cent. of the total protein contents of the kidney was washed out, or more than three times as much as in the previous 147 hours. The erepsin showed a still more remarkable degree of disruption from the tissues, as 58 per cent. of the total amount present in the kidney broke away during the first hour of chloroform perfusion, or the actual rate of disruption was 13,000 times more rapid than during the 3rd to 12th hours of

perfusion. The only other agent found to be comparable to these anæsthetics in disruptive power was free ammonia.[1] Thus perfusion of a kidney for twelve hours with saline containing ·005 to ·025 per cent. of ammonia caused 29 to 35 per cent. of the total protein contents of the tissues to be washed out.

It has been pointed out to me by Dr W. M. Bayliss that bodies like chloroform are known to increase the permeability of the cell-limiting layer. Hence the substances escaping from the cells may not be actually split off from combination with the protoplasm, but merely be enabled to pass out owing to the cell walls being rendered permeable to them. However, this criticism would not apply, as far as we know, to the experiments in which the kidney was perfused with dilute ammonia, or with saline solutions of different strengths, and yet in their case the disintegration was almost as great, and as rapidly induced.

In these experiments the protein washed out from the kidney was estimated by a colorimetric method dependent on the biuret test, whilst the total nitrogen was estimated by Kjeldahl's method. The values so obtained, multiplied by 6·25 to bring them to terms of protein, are larger than the biuret protein values. That is to say, some of the nitrogen was washed out of the kidney in a non-protein form. The amounts so washed out in the above experiment are given in the last column of the table, and it will be seen that they are quite independent of the actual protein values. Though between the 150th and 168th hours of perfusion the protein broke away fifty times more rapidly on an average than between the 3rd and 48th hours, the non-protein nitrogen broke away three times more slowly. It owes its origin to the autolytic action of the intracellular proteolytic enzymes, and hence its amount gradually diminishes during the course of a perfusion, according as less and less of the enzymes and of proteins upon which they can act are left in the tissues. If putrefaction were prevented, it was found that whatever the conditions of perfusion the amount of this autolytic nitrogen remained fairly constant, being on an average about ·05 gm. per hour per kilogram of kidney (expressed in terms of protein). If, how-

[1] Vernon, *Journ. Physiol.*, 35, p. 82, 1906.

ever, the kidney were perfused with saline containing ·01 to ·2 per cent. of lactic acid, this non-protein nitrogen was increased two- to ten-fold, and in fact considerably more than half of the total nitrogen came away from the kidney in a non-protein form. This is an important point, as it shows that a large fraction of the tissue protein is present, not as actual protein, but as potential protein. Generally speaking, this unstable potential protein breaks away as actual protein, and when liberated it becomes quite stable, and is not hydrolysed by dilute lactic acid saline even in the course of several months. The fact that under the influence of dilute lactic acid it readily breaks away as non-biuret-test-yielding substances, shows that the bonds uniting the numerous amino-acid groupings which together constitute a potential protein molecule are very much weaker than they are in free protein molecules. Such looseness of union of the amino-acid groups, and ease of disruption, suggests a corresponding ease in their synthetic combination in the tissues.

The average value given above for the protein autolysed by the perfused kidney, viz. ·05 gm. per hour per kilogram, would come to 84 gm. per day per 70 kg. It is less, therefore, than the protein metabolism of an average man, and much less than that of a smaller animal. If the perfusions had been carried out at 37° instead of at 15° to 20°, the rate of autolysis would have been at least four times greater, but making every allowance for the temperature factor, it must be admitted that in an intact perfused organ the autodigestion after death is not extravagantly greater than that which occurs during life. As long as the endoenzymes remain bound up in the tissues, even if the tissue cells be dead, it is probable that they cannot exercise their digestive powers to a much greater extent than in the living cells. Presumably they can only act upon protein groups which are anchored on to the biogens in their immediate neighbourhood. Once they have broken free of their bonds, however, they are able to attack any or all of the protein groups present in the tissues. Evidence bearing upon this hypothesis has been obtained by Miss Lane-Claypon and Dr Schryver[1] in their autolysis experiments. The liver or

[1] Lane-Claypon and Schryver, *Journ. Physiol.*, 31, p. 169, 1904; Schryver, *ibid.*, 32, p. 159, 1905.

other organ examined by them was removed directly after death, chopped up with a knife, and incubated with saline. Two to twenty-four hours later samples were precipitated with hot trichloracetic acid, and the estimations of the nitrogen in the filtrates showed the amounts of tissue proteins which had arrived at or beyond the peptone stage. It was found that during the first two to four hours there was comparatively little autolysis. For the next six or eight hours it became rapid, and then gradually slowed down. The actual change was considerable. In one liver autolysis, for instance, 13·2 per cent. of the total nitrogen was initially present in the form of peptones: 13·8 per cent. was so present after four hours' incubation: 42·7 per cent. after eight hours, and 63·2 per cent. after twenty-four hours. The initial latent period must be taken to indicate that at first most of the proteolytic endoenzymes were still bound up in the tissues, and so were incapable of exercising more than a very limited activity. Once they were free, however, they digested the tissue proteins at many times their previous rate Taking an average of the values obtained by Lane-Claypon and Schryver in the ten comparable experiments made upon liver autolysis, I find that during the first two hours the autolysis was ·5 unit per hour; during the next two hours, 1·2 units; during the next six hours, 2·87 units; and during the next fourteen hours, ·87 unit.

The protein side-chains are doubtless bound up to the biogens in different ways and with different degrees of firmness. I obtained[1] a direct proof of this by comparing the rate at which the endoerepsin breaks away from the tissues under various experimental conditions with that at which the general mass of protein groups breaks away. On changing the salinity of the liquid with which a kidney was perfused from 1 per cent. to 4 per cent. or *vice versa*, I found that the rate of disruption might be suddenly increased sixty-fold, and it increased to the same extent for both the erepsin and the protein. On the other hand, if already perfused saline were sent through the kidney a second or third time, the protein disruption diminished considerably (*e.g.* to a seventh its previous value), whilst the ferment disruption increased as much as twenty-fold. That

[1] Vernon, *Zeit. f. allgem. Physiol.*, 6, p. 393, 1907.

is to say, one and the same change of condition caused diametrically opposite results in different side-chains. Even the different enzymes are bound up in the tissues with very different degrees of firmness. Thus I found[1] that on extracting minced pancreas, the diastatic enzyme was removed far more readily than the trypsinogen. In one experiment, in which the gland substance was shaken up for two hours with dilute alcohol, 55 per cent. of the total amount of diastase present passed into solution, but only 14 per cent. of the total trypsinogen.

It will be seen that the direct chemico-physical method of studying the tissues during and after death is able to afford some information as to the probable constitution of the biogens during life, and hence it should be pursued simultaneously with the indirect biological method which has yielded such remarkable results during the last decade. It would be out of place in this lecture for me to attempt even a brief summary of the chief conclusions which have been deduced from the vast body of experimental data we possess upon Immunity and allied subjects, hence I will only refer to such portions as bear more especially upon the question of endoenzymes.

The endoenzymes appear to be bound up in the tissues in somewhat the same way as the side-chain receptors. As a rule they are fixed with sufficient firmness to prevent them from being cast off into the blood stream in other than small amounts. But some of them such as maltase are liberated in larger quantities, for the plasma is richer in this enzyme than are any of the tissues. Exoenzymes, as we know, are liberated in large amounts whenever they are required. Probably the endoenzymes and the exoenzymes are formed and are bound up in the tissues in a similar manner, only the linkage binding the exoenzymes is more readily snapped under an appropriate chemical or nervous stimulus than that binding the endoenzymes. If minced pancreatic tissue be extracted with glycerin, the endoenzymic erepsin seems to pass into solution as readily as the exoenzymic trypsin and amylopsin.

We have seen that under conditions of greater functional activity endoenzymes are elaborated and stored up in the

[1] Vernon, *Journ. Physiol.*, 28, p. 466, 1902.

tissues in increased quantity. That is to say, the tissues are capable of over-regenerating their endoenzyme receptors, in somewhat the same way as they regenerate receptors to which toxin molecules have become anchored. Still they do not, as a rule, over-regenerate them to such an extent that they are cast off in a free state into the blood, after the manner of antitoxin molecules. Upon the mechanism of regeneration of receptors, the side-chain theory tells us nothing. Presumably the tissues build them up synthetically by means of their series of proteolytic enzymes. The blood stream contains small quantities of all the various amino acids of which a protein is constituted, and the endoenzymes of the biogens must seize upon these amino acid molecules one by one, as they are required, and build them up together in accordance with some definite pattern which is already laid down in the tissues. Failing such a pattern protein molecule to model fresh receptors upon, it seems likely that the synthesis of the particular kind of receptor in question is impossible. Thus we saw in a previous lecture that certain enzymes such as lactase and invertase were localised in a definite region of the body, viz. the mucous membrane of the alimentary canal, and that if the cells of this membrane lost their power of secreting lactase, they were unable to recover it when subsequently stimulated to do so by a milk diet.

The action of enzymes may be regarded as similar to that of Ehrlich's "receptors of the second order." Each of these receptors has a haptophoric group, which is supposed to anchor on to a suitable receptor in the corpuscle or bacterium it is going to agglutinate. The agglutinophoric group of the receptor then acts upon the cell, and causes the cells to clump together. Similarly, an enzyme attaches itself to a food particle by means of what may be termed its haptophore group, and acts upon it by what may be termed its zymophore group. Endoenzymes are directly comparable to the agglutinating receptors which are bound up in the tissue biogens, whilst exoenzymes are comparable to the receptors which are over-regenerated and cast off into the blood stream when the blood of one animal is injected from day to day into another animal.

In some cases it appears that enzymes act in the same way

as Ehrlich's receptors of the third order. That is to say, they do not bind themselves directly to the food particle upon which they are acting, but only through the intermediation of a third substance or amboceptor. This amboceptor is comparable to the thermostable "immune body" of Ehrlich, which is thrown off by the tissue receptors into the blood of an animal on repeated injection of a foreign blood, and which is able to bind itself on the one hand to a suitable receptor of a blood corpuscle, and on the other hand to some of the thermolabile complement which is always present in the blood, and so enable this complement to produce hæmolysis of the corpuscle. The researches of Fuld, Morawitz, and others[1] show that the conversion of fibrinogen into fibrin is effected by a ferment produced by the interaction of a thrombokinase derived from the leucocytes and other cells with a thrombogen and calcium salts present in the blood plasma. E. W. A. Walker[2] found that oxalate plasma which had been heated for two hours to 50° did not coagulate on the addition of calcium chloride solution, but did coagulate if fresh tissue extract were added as well. This seems to indicate that a temperature of 50° slowly destroys a thermolabile thrombokinase which is present in the oxalate plasma, but does not affect a thermostable thrombogen. Consequently the addition of calcium salts and of fresh thrombokinase (in the tissue extract), brings about coagulation. Though there is no direct proof that thrombogen resembles immune body in binding itself to the fibrinogen on the one hand and to the thrombokinase on the other, yet the fact that both it and immune body are thermostable, whilst both thrombokinase and complement are thermolabile, suggests a complete analogy.

In a similar manner Walker found that ptyalin solutions, inactivated by heating to 50° to 53°, could be re-activated by the addition of blood. This seems to show that ptyalin consists of a specific thermostable substance, without independent activity, and a non-specific kinase or complement, which is present in blood and tissue extracts as well as in saliva. Again, he found that rennet preparations could be inactivated by heating

[1] For literature, see Buckmaster, *Science Progress*, 2, p. 51, 1907.

[2] E. W. Ainley Walker, *Journ. Physiol.*, 33, Proc. xxi., 1905.

to 55°, and re-activated by the addition of fresh liver extract. Donath[1] worked with pancreatic steapsin, and he found that the enzyme was inactivated by heating to 60° to 63°, but could be partially re-activated by the addition of normal horse serum. If the steapsin were heated to 77° to 80°, it could not be re-activated.

However, the activity of steapsin appears to be controlled by still a third factor. Magnus[2] found that liver extracts lost their hydrolytic action upon amyl salicylate when they were dialysed, but that they regained it on addition of boiled liver extract. Hence he thought that the activity of the enzyme depended on a diffusible "co-enzyme." Loevenhart[3] confirmed this result, and he proved the co-enzyme to consist of bile salts. Again, v. Fürth and Schütz[4] found that the action of steapsin on olive oil might be increased fourteen-fold by the addition of bile. Donath observed an even greater effect than this with bile salts, and he concluded that the bile salts liberated free steapsin from a zymogen precursor.

If further experiment confirms the results of Walker and Donath, and the interpretation put upon them, it does not necessarily follow that *all* ferments act in a similar manner through the intermediation of a specific amboceptor or immune body. Some may act directly and some indirectly in the same way as Ehrlich's receptors appear to do.

The existence of separate haptophorous and zymophorous groups in enzyme molecules is supported by other evidence. Korschun[5] found that if rennin solutions were filtered through a Berkefeld filter, the enzyme lost its milk-curdling power to a much greater extent than its power of neutralising antirennin. In fact the strength of the one property might be reduced ten times more than that of the other. This seems to show that the rennin solution consisted partly of ferment molecules with both haptophorous and zymophorous groups, and partly of molecules with haptophorous groups alone, and that the pores

[1] Donath, *Hofmeister's Beitr.*, 10, p. 390, 1907.
[2] Magnus, *Zeit. f. physiol. Chem.*, 42, p. 148, 1904.
[3] Loevenhart, *Journ. Biol. Chem.*, 2, p. 391, 1907.
[4] v. Fürth and Schütz, *Hofmeister's Beitr.*, 9, p. 28, 1906.
[5] Korschun, *Zeit. f. physiol. Chem.*, 37, p. 366, 1903.

of the filter retained a larger proportion of the former molecules than of the latter. Pollak[1] and Schwarz[2] obtained confirmatory evidence by another method. Pollak found that if trypsin solution were inactivated by heating to 70°, it was still able to retard the action of fresh trypsin upon gelatin, and to a much smaller extent, its action upon serum proteins. Similarly, Schwarz found that if pepsin solution were inactivated by heating to 60°, it had a paralytic effect upon the activity of fresh pepsin solutions. Korschun's results indicate that the inhibitory substances, the zymoids[3] or fermentoids as they have been termed, are present, preformed in the original enzyme solution. Hence the destruction of the trypsin or pepsin molecules by heat serves merely to unmask these zymoids.

It was found by Donath[4] that pancreatic steapsin, if inactivated by heating to 70° to 100°, exerted a paralytic effect on active enzyme to which it was added. Again, Bearn and Cramer[5] found that solutions of rennin, taka-diastase, and emulsin, inactivated by heating to 56° to 60° for about half an hour, likewise exerted an inhibitory influence on the activity of the corresponding enzymes, but it was necessary to add a considerable amount of the inactivated enzyme to produce an effect. Different preparations of the same enzyme varied considerably in their inhibitory power, and in some cases gave absolutely negative results. But such failures do not disprove the validity of the positive results. The zymoid molecules may be thrown off by the cells in varying proportions as compared with the enzyme molecules, or the heat inactivation may have destroyed larger or smaller numbers of them in different cases. Though Korschun's results indicate the presence of preformed zymoids, they do not exclude the possibility of conversion of enzyme into zymoid by heat inactivation, or during the gradual deterioriation of activity which all enzyme solutions undergo in course of time. Arguing from the analogy of toxoid formation from toxins, such a conversion is highly

[1] Pollak, *Hofmeister's Beitr.*, 6, p. 95, 1904.
[2] Schwarz, *ibid.*, 6, p. 524, 1905.
[3] *Cf.* Bayliss, *Arch. d. Sci. biol.*, 11, Suppl., p. 271.
[4] Donath, *loc. cit.*
[5] Bearn and Cramer, *Biochem. Journ.*, 2, p. 174, 1907.

probable. Thus Ehrlich observed that the poisonous action of toxin solutions might deteriorate rapidly, whilst their power of binding antitoxin remained nearly constant. For instance, a diphtheria toxin, after being kept for nine months at room temperature, was found to have lost two-thirds of its toxicity, but to have retained its original capacity for binding antitoxin. Ehrlich explained this result by supposing that the toxophorous affinities of the toxin molecules had been destroyed, whilst the haptophorous affinities remained intact.

It seems probable, therefore, that enzyme and zymoid combine with substrate in somewhat the same way that toxin and toxoid combine with antitoxin. Proof of such combination was obtained by Bearn and Cramer in the case of rennin zymoid. They found that if active rennin solution were added to the diluted milk *before* the inactivated rennin, no retardation whatever was produced; but if the inactivated rennin were added before the active enzyme, the coagulation time was nearly doubled. If the inactivated rennin were allowed to stand with the milk at room temperature for five minutes before the addition of the active rennin, the coagulation time was twice as long as when the active rennin was added immediately after the inactive rennin. These experiments indicate that the rennin zymoid gradually combines with some of the caseinogen of the milk and so prevents or delays the action of the rennin enzyme upon it. They require repetition and confirmation, however, as Bearn and Cramer state that their results were irregular.

Antiferments. — Enzymes closely resemble toxins in still another respect, viz. in their power of stimulating the tissues to form anti-bodies. In 1899 Morgenroth[1] showed that the subcutaneous injection of small doses of rennet ferment immunised an animal against the ferment, and its blood serum was found to contain an antirennin. Immune serum of moderate strength was obtained. Thus in one experiment the addition of 2 per cent. of it to milk was sufficient to necessitate the addition of 1 part of rennet ferment in 15,000 before coagulation was induced. In the absence of antiferment, only 1 part of rennet in 3,000,000

[1] Morgenroth, *Cent. f. Bact.*, 26, p. 349, 1899; 27, p. 721, 1900.

was necessary, or 200 times less. The antirennin was specific to the extent of being unable to neutralise vegetable rennet ferment, and this ferment similarly formed an anti-body which could not neutralise rennin of animal origin. Korschun[1] states that the relation of rennin to antirennin closely obeys the laws of toxin-antitoxin reaction, and he finds that at room temperature the union of rennin and antirennin is completed in fifteen minutes. However, Fuld and Spiro[2] state that ordinary blood serum contains rennin in addition to the antirennin previously shown to exist in it by Morgenroth, and that the ferment and its anti-body can be separated by fractional precipitation with ammonium sulphate. The addition of 28 to 33 per cent. of this salt to horse's serum throws down the rennin along with the euglobulin, whilst 34 to 46 per cent. of the salt throws down antirennin along with pseudo-globulin. It seems difficult at first sight to reconcile these statements with those of Korschun, but perhaps ferment and antiferment react with one another in the same way as a weak acid and weak base. As already mentioned in a previous lecture, Arrhenius and Madsen[3] showed that if ammonia and boric acid were allowed to interact, the affinity of the acid and the base for one another is so small that the solution contains, in addition to ammonium borate, considerable quantities of free base and free acid. It seems probable that in the same way mixtures of toxin and antitoxin solutions contain free molecules both of toxin and antitoxin, in addition to the loose toxin-antitoxin combinations. The ratio of free molecules to combined molecules varies with the affinity which the reacting bodies have for one another, and if ferment and antiferment have but a weak affinity, a solution of the two of them would contain sufficient free molecules to render a partial separation possible by fractional salting out.

The attraction of ferment for antiferment and of toxin for antitoxin is probably complicated by a physical factor, dependent

[1] Korschun, *Zeit. f. physiol. Chem.*, 36, p. 141, 1902.

[2] Fuld and Spiro, *ibid.*, 31, p. 132, 1900.

[3] Arrhenius and Madsen. See Arrhenius, *Immuno-chemistry*, New York, 1907, p. 174; also, article by J. Ritchie in *Allbutt and Rolleston's System of Medicine*, 2, Part I., p. 69, 1906.

on their colloidal or semi-colloidal nature. Craw[1] found that the reaction of the lysin and antilysin of *Bacillus megatherium* does not obey the law of mass action, and so he concludes that it is not a purely chemical change. It seems in many ways analogous to the adsorption phenomena mentioned in a previous lecture, but at present there is not sufficient evidence to enable us to decide as to the relative degrees of importance to be attached to the physical and the chemical factors of the interaction.

Rennin is by no means the only ferment for which an antiferment has been obtained. Sachs[2] immunised geese against pepsin, and the serum of these animals contained so much antipepsin that in the presence of 1 c.c. of the serum twenty times more pepsin was necessary to liquefy gelatin than in the presence of 1 c.c. of normal goose serum. Achalme[3] injected sterile pancreatin preparations which had been filtered through a clay filter into the peritoneal cavity of guineapigs, and obtained a fairly active antitryptic serum. Camus and Gley,[4] Landsteiner,[5] and others have shown that normal blood serum contains an antitrypsin. It is attached to the albumin fraction of the serum proteins. Hedin[6] found that if trypsin and serum albumin were mixed together before they were added to the substrate (caseinogen), the neutralising effect of the anti-body was much larger than if they were added separately. If they were allowed to stand together at room temperature for an hour or two before adding them to the substrate, the neutralising effect was larger still. Hence the ferment united or reacted with the anti-body rather slowly, and, as far as one can judge from the data obtained, its affinity for anti-body seemed to be no greater than its affinity for caseinogen. Egg albumin has a much greater antitryptic effect than serum albumin, for I found[7] that the addition of

[1] Craw, *Proc. Roy. Soc.*, B. 76, p. 179, 1905; *Journ. Hygiene*, 7, p. 501, 1907. See also, Arrhenius, *ibid.*, 8, p. 1, 1908.

[2] Sachs, *Fortschr. d. Med.*, 20, p. 425, 1902.

[3] Achalme, *Ann. de l'Inst. Pasteur*, 15, p. 737, 1901.

[4] Camus and Gley, *C. R. Soc. Biol.*, 47, p. 825, 1897.

[5] Landsteiner, *Centralb. f. Bact.*, 27, p. 357, 1900.

[6] Hedin, *Journ. Physiol.*, 32, p. 390, 1905; *Biochem. Journ.*, 1, p. 474, 1906.

[7] Vernon, *Journ. Physiol.*, 31, p. 355, 1904.

1 part of egg albumin to 6000 of trypsin solution reduced the digestive action of this ferment upon fibrin to about half the normal value. Exposure of a solution of egg albumin to a temperature of 60° did not diminish its antitryptic influence, and even after keeping it for three hours at 100° C. the coagulated albumin still retained a good deal of inhibitory power. It is therefore of quite a different nature from the antitrypsin formed by injecting animals with trypsin, for this body is weakened by heating to 56°, and destroyed by heating to 64°.

Antiferments against urease, tyrosinase, laccase, and fibrin ferment have been obtained: also, though not with much certainty, against diastase. Bertarelli[1] immunised rabbits with the lipase of castor-oil seeds, and found that the serum contained an antilipase. This anti-body was active against castor-oil seed lipase, but not against lipases of animal origin. Bertarelli did not succeed in obtaining an antilipase on injecting rabbits and dogs with animal lipases, but this negative result may well have been due to technical difficulties. It seems probable that every enzyme, if injected under suitable conditions into a animal, is able to induce the formation of a specific anti-body. This is another argument in support of the protein-like nature of enzymes, for as far as we know proteins are the only substances to the stimulus of which the cellular protoplasm reacts in this way.

Constitution of Biogens.—Of the constitution of biogens or biogen nuclei we know nothing. Indeed it is doubtful whether an actual nucleus, in the ordinary acceptation of the term, exists at all. All that we know of the constitution of protoplasm concerns only the numerous intracellular enzyme groups described in the previous lectures, and the still more numerous toxin, antitoxin, lysin, agglutinin, precipitin, opsonin, and other similar bodies which we know to exist in the protoplasm, or to be capable of formation by it. Almost every essential constituent of the protoplasm is probably, therefore, a body of protein-like nature. These various protein molecules doubtless differ considerably from one another in size. For instance, Arrhenius and Madsen[2] found that diphtheria antitoxin diffused nearly

[1] Bertarelli, *Centralb. f. Bact.*, 40, p. 231.

[2] Arrhenius and Madsen. See Arrhenius, *Immuno-chemistry*, p. 25.

ten times more slowly than diphtheria toxin, and Craw[1] found that a lysin from *B. megatherium* could pass through a gelatin filter, whilst the antilysin could not. But none of these protein bodies appear to be more complex than the other proteins known to us, and hence their isolation in a pure state, and the determination of their exact chemical composition and even of their chemical constitution, appears to be only a question of time. What is the fundamental difference, therefore, in the structure of a biogen, a unit of living protoplasm, and of the protein molecules of which it consists? We know that it is in an extremely unstable condition, and is continually undergoing decomposition changes and recomposition changes, but does such instability and continual chemical change imply the existence in the biogen of some central nucleus of an entirely different chemical constitution from that possessed by proteins? It might be thought that a complex iron-containing nucleoprotein molecule forms the central nucleus to which numerous protein side-chains are attached, and perhaps such nuclei as this are actually present in the (morphological) nucleus of the cell, which we know to be rich in iron and phosphorus compounds. But the cytoplasm of many cells is extremely poor in organically bound phosphorus. By a microchemical method Macallum[2] showed the presence of small quantities of organic phosphorus in the cytoplasm, but Scott[3] states that the principle of the reaction used is wrong, and that deductions from it as to the distribution of organic phosphorus compounds in cells are valueless. Burian and Walker Hall[4] found that whilst 100 gm. of thymus contain ·40 gm. of purin nitrogen bound up as nucleoprotein, 100 gm. of muscle contain only ·015 gm. Most of this purin must be localised in the nuclei of the muscle fibres, hence it is possible that the cytoplasm of the muscle cells and likewise of many other cells contains no organically bound phosphorus whatever.

[1] Craw, *Proc. Roy. Soc.*, B. 76, p. 179, 1905.

[2] Macallum, *Proc. Roy. Soc.*, 63, p. 467, 1898.

[3] Scott, *Journ. Physiol.*, 35, p. 119, 1906. See also Bensley, *Biol. Bulletin*, 10, p. 49, 1906; Nasmith and Fidlar, *Journ. Physiol.*, 37, p. 278, 1908; and Macallum, *Ergebuisse der Physiol.*, vii., p. 637, 1908.

[4] Burian and Walker Hall, *Zeit. f. Physiol. Chem.*, 38, p. 336, 1903.

In the absence of a nucleoprotein nucleus, it seems to me that the biogens should be regarded rather as a congeries of numerous protein-like molecules, of different chemical constitution and functions, which are loosely bound together by weak chemical bonds. The number of such protein groups in a single biogen is so large that it is impossible for them to hold together as a stable unit. Hence they are continually breaking away and uniting to some neighbouring biogen, or uniting together in some other combination to form a new biogen. Or one might even say that no really isolated biogen units exist at all, but that the protoplasmic contents of a cell consist of a mass of protein-like groupings, loosely bound together, but continually breaking away from one another and uniting together again in fresh combinations and arrangements. Every protein constituent of a biogen is therefore a side-chain, and the central nucleus of a biogen consists only of the general mass of side-chains to which the particular side-chain under consideration is attached. If the cytoplasm of most cells possess no fixed structure or definite stable nuclei, it seems to follow that it cannot perform synthetic functions, and elaborate toxin, enzyme, and other groups from the individual amino acids brought to it in the blood plasma. The evidence, so far as it goes, seems to point to this being the case, for it is well known that enucleated pieces of protoplasm of certain protozoa are unable to assimilate food, or show other synthetic powers, and so speedily die. If the synthetic properties of living tissues be confined to the nuclei of the cells, therefore, it is probable that such synthesis can be induced only by nucleoprotein-containing biogens of definite stable structure. However, in the present state of our knowledge, such discussion is of little value. I have brought forward these suggestions chiefly to indicate that the biogen nucleus is only a theoretical conception, which has at present very small experimental support.

If it be admitted that we have no grounds for assuming that protoplasm contains chemical units of much greater complexity than the protein and nucleoprotein substances known to us, it follows that we must to some extent revise our ideas concerning the anabolic and katabolic processes of living tissues. It has frequently been assumed that protoplasm

is of such enormous complexity that the ordinary protein molecule, as known to us, is but a short stage in the chain of synthetic processes required to elaborate actual living substance; and correspondingly, that the enzymes and other protein-like bodies formed and secreted by living substance represent one of the later stages of protoplasmic katabolism. On the view above suggested, protein-like groups represent practically the summit of synthetic processes. Once they are formed, they loosely combine with other similar protein groups, but the bonds so uniting them are probably of a weaker nature than the bonds uniting the individual amino-acid molecules of which they are composed. They still retain more or less of their own individuality and constitution, therefore, and so should be regarded as the actual working units of the living substance.

It has already been pointed out that if an organ like the kidney be perfused with saline for some days after death, the endoenzymes and proteins of the tissues only very gradually break away from the gland cells, and pass into solution. This might be held to indicate that these products were formed by a gradual breaking down of very complex precursors into simpler and simpler substances, which finally arrived at the stage in which they passed into solution.[1] Though such an explanation cannot be disproved, it is equally probable that the gradual liberation of the enzyme and protein groups is dependent partly on their colloidal nature, and partly on their being bound up in the tissues with various degrees of firmness. We have seen that in the pancreatic tissue the diastatic enzyme is less firmly bound up than the tryptic-rennetic enzyme. Also we know that colloidal bodies react very slowly in combining with or in breaking free from one another, and that their interactions are greatly influenced by physical considerations.

In the case of pepsin, trypsin, and some other ferments, we know that the enzyme passes out from the cell in zymogen form, and that only after such secretion does it become converted into free enzyme. But even this conversion seems to be merely some molecular transformation, and does not involve a breaking down of larger molecules into smaller ones.

[1] *Cf.* Langley, *Journ. Physiol.*, 3, p. 290, 1882.

Thus I found[1] that the precipitability of trypsinogen from a glycerin solution by various strengths of alcohol was practically identical with the precipitability of the free trypsin.

Action of Antiseptics.—The argument that living tissues differ from dead tissues and their disintegration products in degree rather than in kind is supported by another entirely different class of evidence, viz. by their reaction to antiseptics. It is frequently supposed that antiseptics have little or no influence upon the activity of enzymes, whilst very small quantities of them are fatal to the vitality of living organisms. Evidence controverting both suppositions has been adduced incidentally in the course of previous lectures, but the subject is one of such importance that it deserves to be treated more in detail.

As already stated in an earlier lecture,[2] Buchner and Rapp divide antiseptics into two classes, viz. those which enter into chemical combination with proteins (and presumably with the protein constituents of living tissues, and with enzymes), and those which do not. In the first class fall salts of the heavy metals such as mercury, silver, and copper, and perhaps the fluorides, arsenites, and cyanides. In the second class come ether, chloroform, toluol, and other similar bodies, most of which have a small solubility in water, and a great one in fats. Of the heavy metal salts, corrosive sublimate is the best known example. Its action upon protozoa is very variable. Bokorny[3] states that a ·005 per cent. solution kills *Paramœcium* and *Vorticella* in six hours, whilst a ·002 per cent. solution kills in two days. Davenport and Neal[4] found that a ·001 per cent. solution killed Stentors in a minute or two, but in a ·0001 per cent. solution they not only lived, but became so acclimatised to the poison that when subsequently placed in a ·001 per cent. solution they survived two or three times as long as unacclimatised Stentors. Algæ are in some respects more sensitive to corrosive sublimate than protozoa, for Bokorny[5] found that

[1] Vernon, *Journ. Physiol.*, 29, p. 318, 1903.
[2] See p. 91.
[3] Bokorny, *Pflüger's Arch.*, 85, p. 257, 1901.
[4] Davenport and Neal, *Arch. f. Entwickelungsmechan.*, 2, p. 564, 1896.
[5] Bokorny, *Pflüger's Arch.*, 108, p. 216, 1905.

Spirogyra was killed by a ·001 per cent. solution in a few hours, whilst some of the cells were killed after twenty-four hours' immersion in a ·0001 per cent. solution. Cultures of the plant in a ·00001 per cent. solution, and in one ten times more dilute, showed distinct changes, and after a few days in a solution ten times more dilute still (1 in 1000 million), the filaments contained a much smaller store of glycogen than those kept in pure water. Certain bacteria are much more resistant, for Koch says that a ·02 per cent. solution of sublimate does not disinfect invariably.

Enzymes are in some instances just as sensitive to the action of corrosive sublimate as living organisms. A ·01 per cent. solution is said [1] to destroy malt diastase in twenty-four hours, whilst a ·02 per cent. solution destroys yeast zymase and maltase in a similar period. On the other hand, ·1 per cent. solution does not affect rennet ferment, though ·2 per cent. delays its action. This variation in sensitiveness seems very large, but it is due in part at least to the different conditions under which the observations were made. The sublimate destroys an enzyme by combining with it, and if the enzyme solution contains protein impurity, it will combine with this instead, in proportion to the amount of it present, and so the enzyme will be to a greater or less degree protected. The ratio between total quantity of protein and total quantity of sublimate present in a given solution is often, therefore, of greater importance than the actual concentration of the salt. Arguing on this principle, first enunciated by Buchner, Bokorny [2] calculated the lethal dose of various poisons for a given weight of yeast cells and of Spirogyra. He found that if 10 gm. of pressed yeast (containing about 1·5 gm. of dry protein) were mixed with 10 c.c. of ·05 per cent. $HgCl_2$, the cells still showed some reproductive activity after twenty-four hours, but if mixed with 20 c.c. of the sublimate solution, they showed none. Hence the lethal dose of sublimate for 10 gm. of yeast is ·005 to ·01 gm. of $HgCl_2$. In a similar manner Bokorny found the lethal dose of sublimate for 10 gm. of Spirogyra (weighed in the moist condition), to be ·0005 to ·00005 gm. This weight of alga contains ·14 to ·28 gm. of protein, or about

[1] Bokorny, *loc. cit.*

[2] Bokorny, *Pflüger's Arch.*, 111, p. 348, 1906.

a tenth as much as 10 gm. of yeast, and hence the smaller lethal dose roughly corresponds with the smaller protein content of the alga.

To silver salts living organisms and enzymes are probably about as sensitive as they are to mercury salts. A ·02 per cent. solution of silver nitrate kills most bacteria: a ·01 per cent. solution destroys malt diastase, yeast maltase, and zymase in twenty-four hours, whilst a ·05 per cent. solution does not injure rennet ferment (Bokorny).

Fluorides and arsenites are not, as a rule, nearly so poisonous as the salts of the heavy metals. A ·1 per cent. solution of sodium fluoride kills algæ in twenty-four hours, and according to Tappeiner and to Loew, a ·01 per cent. solution prevents putrefaction. Bokorny found that if 10 gm. of yeast were mixed with 50 c.c. of ·1 per cent. NaF, the cells still retained their reproductive activity twenty-four hours later. They were unable to resist twice this quantity of fluoride, however, so the lethal dose of the salt is ·05 to ·1 gm., or ten times greater than that of corrosive sublimate. Upon enzymes fluorides are very much less poisonous than upon living organisms. The most sensitive of them, zymase, has its action stopped by a ·55 per cent. solution of ammonium fluoride, but most other enzymes can act fairly well in the presence of 1 per cent. or more of NaF. Still they are retarded to some extent. For instance, I found[1] that intestinal erepsin took seventeen and a half hours to split up half of the peptone in a given sample when acting in presence of toluol: twenty-six hours when in presence of chloroform, and forty-two hours when in presence of 1 per cent. NaF. Also it is to be remembered that though living organisms cannot exist permanently in more than very dilute fluoride solutions, yet their vitality is not immediately destroyed by concentrated solutions. A 1 per cent. NaF solution takes two hours to kill frog's nerves,[2] and I found[3] that on perfusion of a mammalian kidney with a similar solution, the gaseous metabolism fell only to a half or third the normal during the next three hours, and did not disappear entirely

[1] Vernon, *Journ. Physiol.*, 30, p. 365, 1903.
[2] Davenport, *Experimental Morphology*, London, 1897, p. 22.
[3] Vernon, *Journ. Physiol.*, 35, p. 77, 1906.

even after three days. Perfusion with 1 per cent. arsenious acid solution gave a similar result.

Formaldehyde is sometimes quoted as a suitable poison for the differentiation of enzymes and living organisms, but probably it is no more efficient than those already quoted. Bokorny states that a ·1 per cent. solution acts fatally upon bacteria and other organisms, whilst a ·005 per cent. solution kills Spirogyra in a few days. Also Borkorny found that 25 c.c. of a ·1 per cent. solution were insufficient to destroy the vitality of 10 gm. of yeast in twenty-four hours, whilst twice this quantity of the solution was more than sufficient. Hence the lethal dose was ·025 to ·05 gm. Some enzymes are almost as susceptible to the action of formaldehyde as living organisms, for the activity of malt diastase is very greatly diminished by exposure for twenty-four hours to ·01 per cent. of it. A ·1 per cent. solution acts injuriously on yeast maltase in twenty-four hours, whilst 1 per cent. destroys it. A ·2 per cent. solution destroys zymase in twenty-four hours, whilst ·5 per cent. hinders rennet ferment, though it does not entirely destroy it. Most resistant of all is the enzyme myrosin, which is not affected by 1 per cent. of formaldehyde, and is destroyed only after twenty-four hours by 5 per cent. of it.[1] Loew found that a 5 per cent. solution rapidly destroyed catalase.

Antiseptics of the second class do not enter into chemical combination with proteins, and their action perhaps depends upon their solubility in the fatty constituents of the cell. Hence, beyond a certain minimal limit, their action should vary only with their concentration, and there should be no quantitative relationship between the amount of protoplasm or protein present and the amount of poison.

Antiseptics of this second class, such as ether, chloroform, thymol, toluol, and phenol, are of much greater value than those of the first class for differentiating between living organisms and enzymes. No organism can live in their saturated aqueous solutions, whilst most enzymes can still exert their activity, often with extremely little retardation. The great sensitiveness of living organisms probably depends upon the disintegrating effect exerted by the antiseptic solutions upon living tissues.

[1] *Cf.* Bokorny, *Pflüger's Arch.*, 85, p. 257, 1901.

As already mentioned, I found that perfusion of a freshly excised mammalian kidney with saturated solutions of chloroform or ether caused an immediate disintegration of the tissues, and the passage outwards, in the perfusion liquid, of large amounts of proteins and endoenzymes. It might be supposed that the action of the antiseptic was first and foremost to kill the cellular protoplasm, and that disruption of the biogens ensued only subsequent to their death, and in consequence of it. In that a similar disintegration of the tissues of a dead kidney occurs immediately it is perfused with ether or chloroform saline, it looks rather as if the action of the antiseptic were directly upon the chemical bonds which unite the protein and enzyme groups to the biogens, and that death of the protoplasm ensued subsequent to the breakage of these bonds, or simultaneously with it.

The number of instances in which the influence of these antiseptics upon enzyme action has been investigated quantitatively is comparatively small. As stated in a previous lecture, Magnus-Levy[1] found that large pieces of liver and other organs underwent more autolysis in a single day at 37° under aseptic conditions than in several months if chloroform or toluol were present. In contrast to this, Lane-Claypon and Schryver[2] found that minced liver, kept in saline at 37°, underwent autolysis almost as rapidly in presence of toluol as in its absence. Buchner found that toluol slightly retarded the action of zymase upon sugar, whilst thymol reduced the CO_2 output by a half. Bokorny[3] states that chloroform does not influence the action of emulsin or of rennin, but that small quantities of it destroy pepsin. On the other hand, a saturated solution of thymol (1 in 1100) destroys rennin. Kastle and Loevenhart[4] found that ·02 per cent. solutions of toluol, chloroform, and phenol hardly affected the action of liver lipase upon ethyl butyrate, whilst thymol and salicylic acid retarded it slightly. In the case of phenol alone of the second class of antiseptics is the action upon enzyme and living organisms

[1] Magnus-Levy, *Hofmeister's Beitr.*, 2, p. 261, 1902.
[2] Lane-Claypon and Schryver, *Journ. Physiol.*, 31, p. 169, 1904.
[3] Bokorny, *Pflüger's Arch.*, 85, p. 257, 1901.
[4] Kastle and Loevenhart, *Amer. Chem. Journ.*, 24, p. 491, 1900.

closely comparable. A ·5 per cent. solution of it kills Anthrax bacilli, but even a 5 per cent. solution does not kill all spores. A ·1 per cent. solution kills yeast cells, but does not affect zymase and maltase. However, these two enzymes are rendered inactive by a 1 per cent. solution, though the invertase which accompanies them does not seem to be affected (Bokorny).

With regard to antiseptics as a whole, therefore, we may say that there is no sharply defined demarcation between the reaction of living organisms and that of enzymes. Living organisms differ more widely amongst themselves in their sensitiveness to antiseptics than do the most resistant organisms from enzymes. A striking instance of this is seen in the case of Anthrax spores just recorded, for they can withstand a concentration of phenol 100 times greater than that which kills most other forms of life.

Influence of Temperature.—The essentially chemical nature of enzyme action, and of the changes occurring in living tissues, is indicated by the response of these processes to temperature changes. Arrhenius[1] and van't Hoff[2] have shown that the velocity of chemical reactions is roughly speaking doubled for each rise of temperature of 10°. The observed quotients vary between the limits of 1·2 and 3·68, but the majority of them vary only from 1·9 to 2·7. On the other hand, purely physical processes such as the electrical conductivity of a wire, the viscosity of a liquid, osmotic pressure and surface tension, are not affected to anything like this extent by rise of temperature.

As can be seen from the data given in the table, the velocity of enzyme action follows the law of chemical reactions, and not that of physical processes. We see that in the case of various proteolytic, milk-curdling, lipolytic, amylolytic, and catalytic enzymes, quotients of about 2 were obtained. In some cases more divergent results have been arrived at, but they are omitted from the table as there seemed in their case good reason to suspect secondary phenomena which modified the primary temperature effects. It will be noted that the upper temperature limit to which the quotient was estimated in no

[1] Arrhenius, *Zeit. f. physik. Chem.*, 4, p. 226, 1899.

[2] Van't Hoff, *Lectures on Theoretical and Physical Chemistry*, London, 1899, I., p. 228.

case exceeds 40°. It has in some instances been determined up to 60° or 70°, but it is usually found that at temperatures above 40° the quotient gets smaller and smaller, and even becomes negative. This is due entirely to the destructive effect of high temperature upon the enzyme. The destruction

Enzyme and Substrate.	Temperature Range.	Quotient for 10°.
Action of Trypsin on Witte's Peptone * . . .	15° to 25°	2·3
„ Erepsin on Witte's Peptone * . . .	15° „ 25°	2·6
„ Pancreatic Rennin on Milk † . . .	20° „ 30°	2·1
„ Gastric Rennin on Milk ‡	25° „ 40°	3·1
„ Amylopsin on Starch (in ·2% NaCl) † . .	20° „ 30°	2·0
„ Castor-oil Bean Lipase on Triacetin § . .	18° „ 28°	2·6
„ Blood Catalase on Hydrogen Peroxide ‖ .	0° „ 10°	1·5

* Vernon, *Journ. Physiol.*, 30, p. 364, 1903. † Vernon, *ibid.*, 27, p. 190, 1901.
‡ Fuld, *Hofmeister's Beitr.*, 2, p. 184, 1902. § Taylor, *Journ. Biol. Chem.*, 2, p. 87, 1906.
‖ Senter, *Zeit. f. physik. Chem.*, 44, p. 257, 1903.

becomes greater and greater the higher the temperature, till at the so-called maximum temperature the enzyme is destroyed almost immediately. The optimum temperature of enzyme action is that temperature at which it acts most rapidly, or at which the increased velocity due to high temperature is just balanced by a diminution of effect resultant on destruction of the enzyme. It is therefore a variable point, dependent on the rate at which the reaction is progressing, and the presence of other substances which may increase or decrease the stability of the enzyme. For instance, Tammann[1] found that when a fixed amount of salicin was acted upon by one part of emulsin, or sufficient to hydrolyse 34 per cent. of it in twenty hours, the optimum temperature was 26°. With two parts of emulsin, the optimum was 35°, and 46·5 per cent. was hydrolysed in twenty hours. With four to sixty-four parts, the optimum was 46°, and 64·0 to 94·5 per cent. was hydrolysed; whilst with 128 parts, the optimum was 54°, and 94·5 per cent. was hydrolysed. Again, the writer[2] found that pancreatic diastase, if allowed to act upon starch paste made up with tap

[1] Tammann, *Zeit. f. physiol. Chem.*, 18, p. 436, 1894.
[2] Vernon, *Journ. Physiol.*, 27, p. 190, 1901.

water, attained its optimum at 35°; but if it were acting in presence of ·2 per cent. NaCl, the conditions were so much better adapted to its activity and stability that at 35° it digested the starch four and a half times more rapidly, whilst at its optimum temperature, viz. 50°, it digested it nine times more rapidly. The maximum temperature in both cases lay between 65° and 70°, whilst that of pancreatic rennet lay at something above 70°. Tammann's results show that the maximum temperature for the action of emulsin on salicin is about 75°, whilst that for yeast invertase on cane-sugar is about 62°. Brown and Heron[1] found that malt extract could be heated to 76°, and the diastase would then act upon starch at 75°, so its maximum temperature must be a little above these figures. Nicloux[2] found that the action of castor-oil seed lipase upon olive oil is destroyed in ten minutes at 55°, hence its maximum temperature is probably about 60°. Donath[3] found that pancreatic steapsin was inactivated at 55° to 65°; Schwarz,[4] that pepsin was inactivated by heating to 60°; Bearn and Cramer,[5] that rennin, pepsin, taka-diastase and emulsin were inactivated at 56° to 60°. Again, Mayer[6] states that pepsin solutions lose their activity when warmed to 57°, but Pekelharing[7] found that exposure of a pepsin solution for two minutes to a temperature of 70° did not entirely destroy the ferment.

We may conclude, therefore, that the great majority of enzymes are destroyed at a temperature varying in different cases from 60° to 77°.

The metabolic changes of living organisms, being of an essentially chemical nature, might be expected to comply with the law observed for chemical reactions and enzyme actions. This is actually the case unless disturbing factors such as nervous control are introduced. As pointed out by

[1] Brown and Heron, *Journ. Chem. Soc. Trans.*, 1879, p. 596.
[2] Nicloux, *C. R. Soc. Biol.*, 56, pp. 701, 839, and 868, 1904.
[3] Donath, *Hofmeister's Beitr.*, 10, p. 390, 1907.
[4] Schwarz, *ibid.*, 6, p. 524, 1905.
[5] Bearn and Cramer, *Biochem. Journ.*, 2, p. 174, 1907.
[6] Mayer, *Zeit. f. Biol.*, 17, p. 351, 1881.
[7] Pekelharing, *Zeit. f. physiol. Chem.*, 22, p. 242, 1897.

van't Hoff,[1] the observations of Clausen[2] upon the carbonic acid discharge of seedlings of wheat, lupins, and syringa flowers show a temperature quotient of 2·5 between 0° and 25°. Miss Matthaei[3] found that the assimilation of carbon dioxide by a leaf of *Prunus Laurocerasus* had a temperature quotient of 2·4 between 0° and 10°; one of 2·1 between 10° and 20°, and one of 1·8 between 20° and 30°. Aberson[4] found that the fermentation of glucose by yeast was 2·9 times more rapid at 27° than at 17·5°. The writer[5] investigated the effect of temperature upon the oxygen intake of various marine animals, and between 10° and 20° obtained quotients of 1·9 to 3·7 for various transparent pelagic Medusæ, Ctenophores and Salpæ, and quotients of 1·6 to 2·2 for various Mollusca and Fishes.

When we pass to higher forms of animal life, we find that the temperature quotient does not by any means correspond with the theoretical law. Not only does it vary considerably in different animals, but in the same animal it shows great differences at different temperature intervals. For instance, in the frog (*R. temporaria*) the writer found[6] it to be on an average 1·8 between 10° and 20°, and 3·5 between 20° and 30°. In the toad it was 1·3 and 4·1 for the same temperature intervals respectively. Other cold-blooded animals showed similar differences, and even the earthworm had quotients of 1·3 and 4·1 for the respective temperature intervals mentioned.

Not only does the general metabolism of living organisms follow the theoretical law, but many other vital processes not necessarily dependent on chemical changes obey it also. Cohen[7] pointed out that the temperature effects observed by Hertwig[8] upon the segmentation of developing frogs' eggs correspond with it. The first stage of development gave a temperature quotient of 2·2, and the later stages, one of 3·3. With Echino-

[1] Van't Hoff, *Vorlesungen*, I., p. 224, 1901.
[2] Clausen, *Landwirtsch Jahrb.*, 19, p. 892, 1890.
[3] Matthaei, *Phil. Trans. Roy. Soc.*, B. 197, p. 47, 1904.
[4] Aberson, quoted from Arrhenius, *Immuno-chemistry*, p. 139.
[5] Vernon, *Journ. Physiol.*, 19, p. 18, 1895.
[6] Vernon, *ibid.*, 21, p. 443, 1897.
[7] Cohen, *Vorlesungen über physikalische Chemie*, p. 42, 1901.
[8] Hertwig, *Arch. f. mikrosk. Anat.*, 51, p. 319, 1898.

derm eggs, Peter[1] obtained a quotient of 2·3 for the first stage, and 2·1 for the later stages. Loeb[2] found that the velocity of artificial maturation of *Lottia* eggs is more than doubled on raising the temperature from 8° to 18°.

Upon the beating heart a number of observations has been made. Snyder[3] observed that the isolated heart of the Pacific terrapin (*Clemys marmorata*) obeyed the law between the temperature limits of 2·5° and 30°. Between 10° and 20° the quotient was 2·7, and between 20° and 30°, 2·2. At 35°, the optimum temperature, the heart beat 48·6 times per minute, but at 40° it sank to 43·3 per minute, so the high temperature acted destructively in the same way as it is observed to do upon enzymes. T. B. Robertson[4] determined the influence of temperature upon the rate of heart beat in the transparent fresh-water crustacean *Ceriodaphnia*, and between the limits of 11° and 29° he obtained an average quotient of 2·03. At 35° the heart stopped permanently. Snyder[5] investigated the transparent nudibranch *Phyllirrhoe*, and he found that between the limits of 16° and 29° the average temperature quotient for the heart beating *in situ* worked out at 2·52. The isolated heart of the crustacean *Maia verrucosa* had a co-efficient of 2·99 between the limits of 7° and 26°. Snyder also pointed out that the observations of previous investigators upon the mammalian heart likewise conform to the law of chemical reaction velocities. Newell Martin[6] experimented on the isolated heart of the dog, and between the limits of 27·8° and 42·5° he obtained quotients varying from 1·8 to 3·3. Martin and Applegarth[7] obtained quotients of 1·3 to 3·2 for isolated cats' hearts between the temperatures of 22·3° and 40·0°. Langendorff[8] succeeded in maintaining the rhythm of isolated cats' hearts between the temperature limits of 7° and 47°, and

[1] Peter, *Arch. f. Entwickelungsmechan.*, 20, p, 130, 1905.
[2] Loeb, "University of California Publications," *Physiology*, 3, p. 1.
[3] Snyder, *ibid.*, 2, p. 125, 1905.
[4] T. B. Robertson, *Biol. Bulletin*, 10, p. 242, 1906.
[5] Snyder, *Amer. Journ. Physiol.*, 17, p. 350, 1906.
[6] N. Martin, *Phil. Trans. Roy. Soc.*, 174, p. 679, 1883.
[7] Martin and Applegarth, *Studies from the Biol. Laboratory, Johns Hopkins Univ.*, 4, p. 282, 1890.
[8] Langendorff, *Pflüger's Arch.*, 66, p. 355, 1897.

from his experimental data Snyder calculated that between the temperatures of 16° and 39° the quotient varied only from 1·8 to 3·7, and in most cases kept at about 2·7. At temperatures above 39° it got less and less, but no actually negative quotient was obtained. The human heart obeys the law no less than that of other mammals, for Davy recorded his temperature and pulse rate three times a day for eight consecutive months, and found that when the mean temperature varied from 36·62° to 37·07°, the mean pulse rate increased from 54·68 to 57·2. Snyder calculated that these data give quotients of 2·3 to 3·1.

These numerous concordant observations upon the heart beat appear to indicate that the beats are due to a constantly repeated chemical reaction. The changes occurring in the heart preparatory to a succeeding contraction are likewise of a chemical nature, for, as Snyder points out, the refractory period of the heart is affected by temperature in the same way as the contraction rate. Burdon Sanderson and Page[1] observed that between 12° and 27° the refractory period in the frog's heart diminished from 2·0″ to ·8″, or gave temperature quotients of 1·8 to 2·5.

More interesting even than the effect of temperature upon heart beat and gaseous metabolism is its influence upon the propagation of the nervous impulse. The resistance of nerve to fatigue, and the apparent absence of chemical changes on stimulation, suggest that the transmission of the nervous impulse is a physical rather than a chemical process. However, the results recently obtained show that temperature influences the propagation rate in the same way that it affects chemical reactions, or that the mechanism of propagation must be of an essentially chemical nature. Nicolai[2] determined the rate of propagation in the olfactory nerve of the pike, and found that at temperatures ranging from 3·5° to 25° the rate varied from 5·65 metres per second to 22·2 metres. From these data Snyder[3] calculated the temperature quotient to vary between 1·8 and 4·1, or to average 2·55. v. Miram[4] determined the

[1] Sanderson and Page, *Journ. Physiol.*, 2, p. 384, 1880.
[2] Nicolai, *Pflüger's Arch.*, 85, p. 65, 1901.
[3] Snyder, *Arch. f.* (*Anat. u.*) *Physiol.*, 1907, p. 113.
[4] v. Miram, *ibid.*, 1906, p. 533.

propagation rate in the motor nerves of the frog between 15° and 35°, and his results give quotients varying from 1·4 to 2·8, and averaging 2·1. Maxwell[1] experimented with the pedal nerves of the giant slug, *Ariolimax columbianus*. The mean impulse rate is only ·44 metre per second, and as it is possible to use a nerve 10 cm. in length, the determination can be made with considerable exactness. The temperature range was −1° to 26°, and the quotients obtained varied from 1·08 to 3·15. The majority of them varied only from 1·5 to 2·1, however, and as they averaged 1·78, we may say that in all the experiments recently recorded the nerve propagation rate was found to be influenced by temperature in accordance with the chemical law.

The fact that many of the vital processes of living cells are of an essential chemical nature does not prove that they are brought about by enzyme action. Indeed, a seeming proof to the contrary is afforded by the fact that enzymes can exert their action at temperatures of 60° to 76°, whilst the vitality of most living cells is destroyed at something under 50°. In the case of the higher vertebrates death may be due to the coagulation of the cell globulins, for extracts of most tissues (*e.g.* liver, spleen, muscle, nerve) have been found[2] to contain a globulin coagulating at 45° to 50°. But some organisms are killed at temperatures considerably below that at which, as far as we are aware, any protein coagulation occurs. I found,[3] for instance, that the ova of the Echinoid *Strongylocentrotus lividus* were killed at 28·5°. After fertilisation their death temperature rose, during the next four hours, to 33·5°, and during the next twenty-four hours (by which time they had reached the pluteus stage) to 39·5°. Hence the cause of death in these developing organisms is absolutely obscure.

Under certain circumstances, however, living organisms can withstand as high a temperature as enzymes. Dallinger[4] gradually acclimatised certain Flagellata to increasing high

[1] Maxwell, *Journ. Biol. Chem.*, 3, p. 359, 1907.

[2] See article by Halliburton in Schäfer's *Text Book of Physiology*, 1, pp. 85, 87, 96, and 118.

[3] Vernon, *Journ. Physiol.*, 25, p. 135, 1899.

[4] Dallinger, *Journ. Roy. Microsc. Soc.*, 7, p. 191, 1887.

temperature, till after about six years they were able to live and multiply at 70°. Various protophyta[1] are known which flourish in hot springs at temperatures as high as 93°. Some spores (*e.g.* Anthrax) can be boiled in water for several minutes without losing their vitality. It may be asked why such high temperatures as these do not cause death by coagulating the cell proteins. The explanation is probably to be found in the fact that the temperature of coagulation of a protein varies inversely with the amount of water it contains. Lewith[2] states that egg albumin coagulates at 74° to 80° when in presence of 25 per cent. of water; at 80° to 90° with 18 per cent. of water; at 145° with 6 per cent. of water, and at 160° to 170° with no water at all. So in all probability organisms capable of withstanding high temperatures contain less water than usual. This is almost certainly the case with spores. In this connection it is interesting to recall the fact that dried enzyme preparations can readily withstand a temperature of 100° or more.

Though the whole of the processes occurring in living cells are certainly not due to the action of endoenzymes, we seem justified in concluding that many or most of the chemical processes are dependent upon them directly or indirectly. Though our knowledge of the subject is at present very incomplete, we have sufficient information to suggest that it is only a question of time and extended research before we shall be able to give final and conclusive answers to many of the questions tentatively raised in the course of these lectures.

[1] For literature see Davenport and Castle, *Arch. f. Entwickelungsmechan.*, 2, p. 227, 1895.

[2] Lewith, *Arch. f. exp. Path.*, 26, p. 341, 1884.

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